IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF COLUMBIA

CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE 3, rue Michel Ange F-75794 Paris Cedex 16, France Plaintiff, v. HON. DAVID J. KAPPOS Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office Madison Building 600 Dulany Street Alexandria, VA 22314 Defendant.	)	Civil Action No. _____

COMPLAINT

Plaintiff Centre national de la recherche scientifique, for its Complaint against Defendant, the Honorable David J. Kappos, states as follows:

1. This is an action by the owner of United States Patent No. 7,521,212 ("the '212 patent") seeking review of erroneous patent term adjustment calculations made by the United States Patent & Trademark Office ("PTO"). Specifically, this is an action by Plaintiff under 35 U.S.C. § 154(b)(4)(A) seeking a judgment that the patent term adjustment of 264 days calculated by the PTO for the '212 patent should be corrected to reflect no less than 839 days of patent term adjustment for the '212 patent.

2. This action arises under 35 U.S.C. § 154 and the Administrative Procedure Act, 5 U.S.C. §§ 701-706.

I. THE PARTIES

3. Plaintiff Centre national de la recherche scientifique ("CNRS") is a company operating under the laws of France. CNRS is located at 3, rue Michel Ange, F-75794 Paris Cedex 16, France.

4. Defendant David J. Kappos is the Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office. Defendant is sued in his official capacity.

II. JURISDICTION AND VENUE

5. This Court has jurisdiction over this action and is authorized to issue the requested relief to Plaintiff pursuant to 28 U.S.C. §§ 1331, 1338(a) and 1361; 35 U.S.C. § 154(b)(4)(A); and 5 U.S.C. §§ 701-706.

6. Venue is proper in this district pursuant to 35 U.S.C. § 154(b)(4)(A).

7. This original Complaint was timely filed in accordance with 35 U.S.C. § 154(b)(4)(A).

III. BACKGROUND

8. The '212 patent issued to Eric Samain and Bernard Priem on April 21, 2009, based on patent application number 10/019,954, which entered the national stage under 35 U.S.C. § 371(c) on May 24, 2002. The '212 patent is attached hereto as Exhibit A.

9. Plaintiff CNRS is the assignee of the '212 patent, as evidenced by records recorded in the PTO, and is the real party in interest in this case.

10. When the PTO issued the '212 patent on April 21, 2009, it erroneously calculated the patent term adjustment for the '212 patent as 264 days. Had the PTO calculated the

patent term adjustment properly, the '212 patent would be entitled to no less than 839 days of patent term adjustment.

11. At least some of the errors in the PTO's patent term adjustment calculation methodology are detailed in a recent order from the U.S. District Court for the District of Columbia in an action titled *Wyeth v. Dudas*, Civil Action No. 07-1492 (D.D.C. Sept. 30, 2008), where the Court granted summary judgment against the PTO, holding that the PTO's patent term adjustment calculation methodology was erroneous as a matter of law and inconsistent with the plain text of the Patent Statute. The *Wyeth* opinion is attached as Exhibit B.

12. The corrected patent term adjustment methodology identified in the *Wyeth* opinion is believed to govern the PTO's calculation of patent term adjustment for Plaintiff's '212 patent (the *Wyeth* opinion is currently being appealed by the PTO to the U.S. Court of Appeals for the Federal Circuit).

IV. COUNT: U.S. PATENT NO. 7,521,212

13. Plaintiff incorporates by reference the allegations in paragraphs 1-12 above, as if fully set forth herein.

14. During prosecution of the '212 patent, the patent owner accrued at least 435 days of patent term adjustment under 35 U.S.C. § 154(b)(1)(A), and accrued at least 782 days of patent term adjustment under 35 U.S.C. § 154(b)(1)(B).

15. Under the PTO's interpretation of 35 U.S.C. § 154, all patent term adjustment ("PTA") accrued under 35 U.S.C. § 154(b)(1)(A) and all PTA accrued under 35 U.S.C. § 154(b)(1)(B) inherently overlaps and, thus, it has been the PTO's position that a patent holder is only eligible for the larger of these two amounts of PTA. For the '212 patent, the PTO erroneously limited the patent term adjustment for the '212 patent to 264 days (*see* calculation in paragraph 22, below), as shown on the face of the '212 patent.

16. In view of a recent decision from the this Court (*Wyeth v. Dudas*, Civil Action No. 07-1492 (JR)), all non-overlapping days on which 35 U.S.C. § 154(b)(1)(A) or 35 U.S.C. § 154(b)(1)(B) apply should accrue patent term adjustment for the '212 patent. As set forth in *Wyeth*, the only way that periods of time can overlap is if they occur on the same day.

17. Calculation under 35 U.S.C. § 154(b)(1)(A): Each day from the day after at least July 24, 2003 (14 months from the National Stage entry (all 371(c) requirements met)) through to the issuance of a Restriction Requirement on October 1, 2004, qualify for patent term adjustment under 35 U.S.C. § 154(b)(1)(A), a total of at least 435 days.

18. Calculation under 35 U.S.C. § 154(b)(1)(B): Each day from the day after at least January 7, 2005 (3 years after the National Stage commencement) through to the filing date of a Request for Continued Examination of February 28, 2007, qualify for patent term adjustment under 35 U.S.C. § 154(b)(1)(B), a total of at least 782 non-overlapping days.

19. Under the interpretation of this Court (*Wyeth v. Dudas*, Civil Action No. 07-1492 (JR)), the non-overlapping days and the total PTO prosecution delay total at least 1,217 days, as shown in the chart attached as Exhibit C.

20. The PTO found a total applicant prosecution delay of 378 days under 35 U.S.C. § 154(b)(2)(B) or (C).

21. It is accordingly believed that the overall PTA accrued by the patent holder is no less than 1,217-378 = 839 days.

22. Under the PTO's interpretation, the PTO calculated an erroneous patent term adjustment of 645-378 = 264 days for the '212 patent. Apart from the errors made by the PTO under the *Wyeth* opinion, the PTO also erroneously calculated the 35 U.S.C. § 154(b)(1)(B) term three years from the National Stage 371(c) *completion* date as opposed to the National Stage 371(c) *commencement* date, which accounts for an additional 137 days of patent adjustment term that should have been awarded to the Plaintiff, as set forth in 1347 *Official Gazette* 49 (Oct. 6, 2009), entitled "Notice Concerning Calculation of the Patent Term Adjustment under 35 U.S.C. § 154(b)(1)(B) involving International Applications Entering the National Stage Pursuant to 37 U.S.C. § 371".

23. The patent holder accordingly requests no less than 839-264 = **575 ADDITIONAL days** of Patent Term Adjustment.

WHEREFORE, Plaintiff respectfully prays that this Court:

A. Issue an Order changing the period of patent term adjustment for the '212 patent term from 264 days to no less than 839 days and requiring Defendant to alter the terms of the '212 patent to reflect the at least 839 days of actual patent term adjustment due the '212 patent.

B. Grant such other and further relief as the nature of the case may admit or require, including additional patent term for the '212 patent if further errors are identified and found in the PTO's patent term adjustment calculation methodology, and any such other and further relief as may be deemed just and equitable by this Court.

Dated: October 19, 2009

Respectfully submitted,

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US007521212B1

EXHIBIT A

(12) **United States Patent**
Samain et al.

(10) **Patent No.: US 7,521,212 B1**
(45) **Date of Patent: Apr. 21, 2009**

(54) **METHOD FOR PRODUCING OLIGOPOLYSACCHARIDES**

(75) Inventors: **Eric Samain**, Gieres (FR); **Bernard Priem**, Grenoble (FR)

(73) Assignee: **Centre National de la Recherche Scientifique (CNRS)**, Paris (FR)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 264 days.

(21) Appl. No.: **10/019,954**

(22) PCT Filed: **Jul. 7, 2000**

(86) PCT No.: **PCT/FR00/01972**

§ 371 (c)(1),
(2), (4) Date: **May 24, 2002**

(87) PCT Pub. No.: **WO01/04341**

PCT Pub. Date: **Jan. 18, 2001**

(30) **Foreign Application Priority Data**

Jul. 7, 1999 (FR) 99 08772

(51) **Int. Cl.**
C12P 19/18 (2006.01)
C12P 19/26 (2006.01)

(52) **U.S. Cl.** **435/84**; 435/97; 435/193; 536/55.2; 536/55.3; 536/123; 536/123.1; 536/124; 536/126

(58) **Field of Classification Search** 435/97, 435/193, 440
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,945,314 A * 8/1999 Prieto et al. 435/101

FOREIGN PATENT DOCUMENTS

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EP	0 315 496	5/1989
EP	0 392 556	10/1990
WO	WO 95 02683	1/1995
WO	96/10086	* 4/1996
WO	WO 98 44145	10/1998

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K.F. Johnson. "Synthesis of Oligosaccharides By Bacterial Enzymes", Glycoconjugated Journal 16(2): 141-146, (Feb. 1999).*
S. Koizumi et al. "Large-Scale Production of UDP-Galactose and Globotriose By Coupling Metqabolically Engineered Bacteria", Nature Biotechnology 16:847-850, (Sep. 1998).*
G.F. Herrmann et al. "Recombinant Whole Cells as Catalysts for the Enzymatic Synthesis of Oligosaccharides and Glycopeptides", Angewandte Chemie International Edition in English 33(12): 1241-1242 (Jun. 6, 1994).*

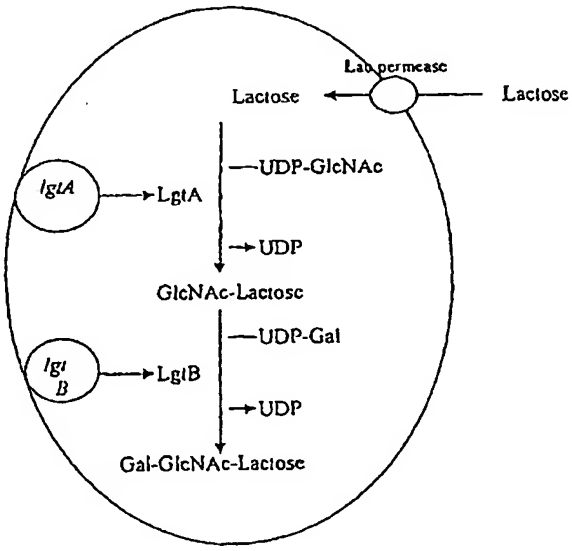
(Continued)

Primary Examiner—Rebecca Prouty
(74) *Attorney, Agent, or Firm*—Foley & Lardner LLP

(57) **ABSTRACT**

The invention concerns the production by microbiological process of oligopolysaccharides of biological interest. More particularly, the invention concerns a method for synthesizing in vivo oligopolysaccharides by internalization of an exogenous precursor in growing bacterial cells expressing adequate modifying and glycosylating genes.

14 Claims, 9 Drawing Sheets



OTHER PUBLICATIONS

Bettler et al., "The Living Factory: In Vivo Production of N-acetyl-lactosamine containing carbohydrates in *E. coli*", Glycoconjugate Journal, 1999, pp. 205-212, vol. 16, XP002134857, Chapman & Hall., GB.

Samain et al., "Production of O-acetylated and Sulfated Chitooligosaccharides by Recombinant *Escherichia coli* Strains Harboring Different Combinations of Nod Genes", Journal of Biotechnology, 1999, pp. 33-47, vol. 72, No. 1-2, XP004172885, Elsevier Science Publishers, Amsterdam.

Plumbridge et al., "Convergent Pathways for Utilization of the Amino Sugars N-acetylglucosamine, N-acetylmannosamine and N-acetyleneuraminic Acid by *Escherichia coli*", Journal of Bacteriology, 1999, pp. 47-54, vol. 181, No. 1, XP000917021.

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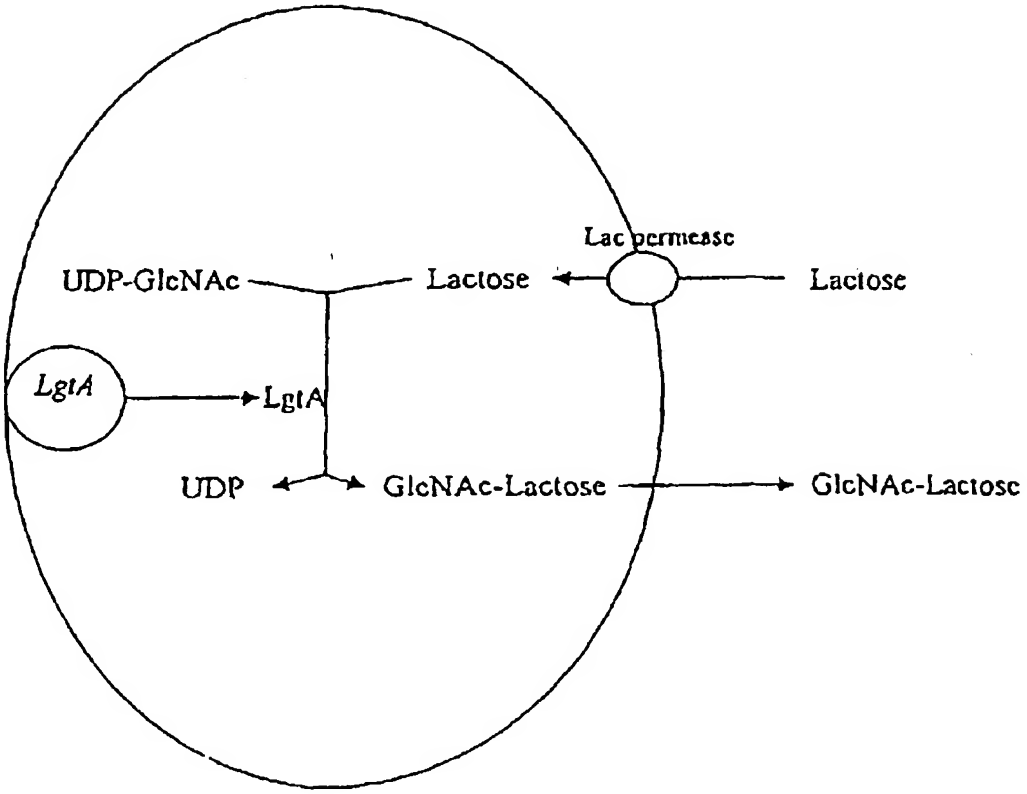
Lac permease
UDP-GlcNAc
Lactose
Lactose
LgtA
LgtA
UDP
GlcNAc-Lactose
GlcNAc-Lactose

FIG-1

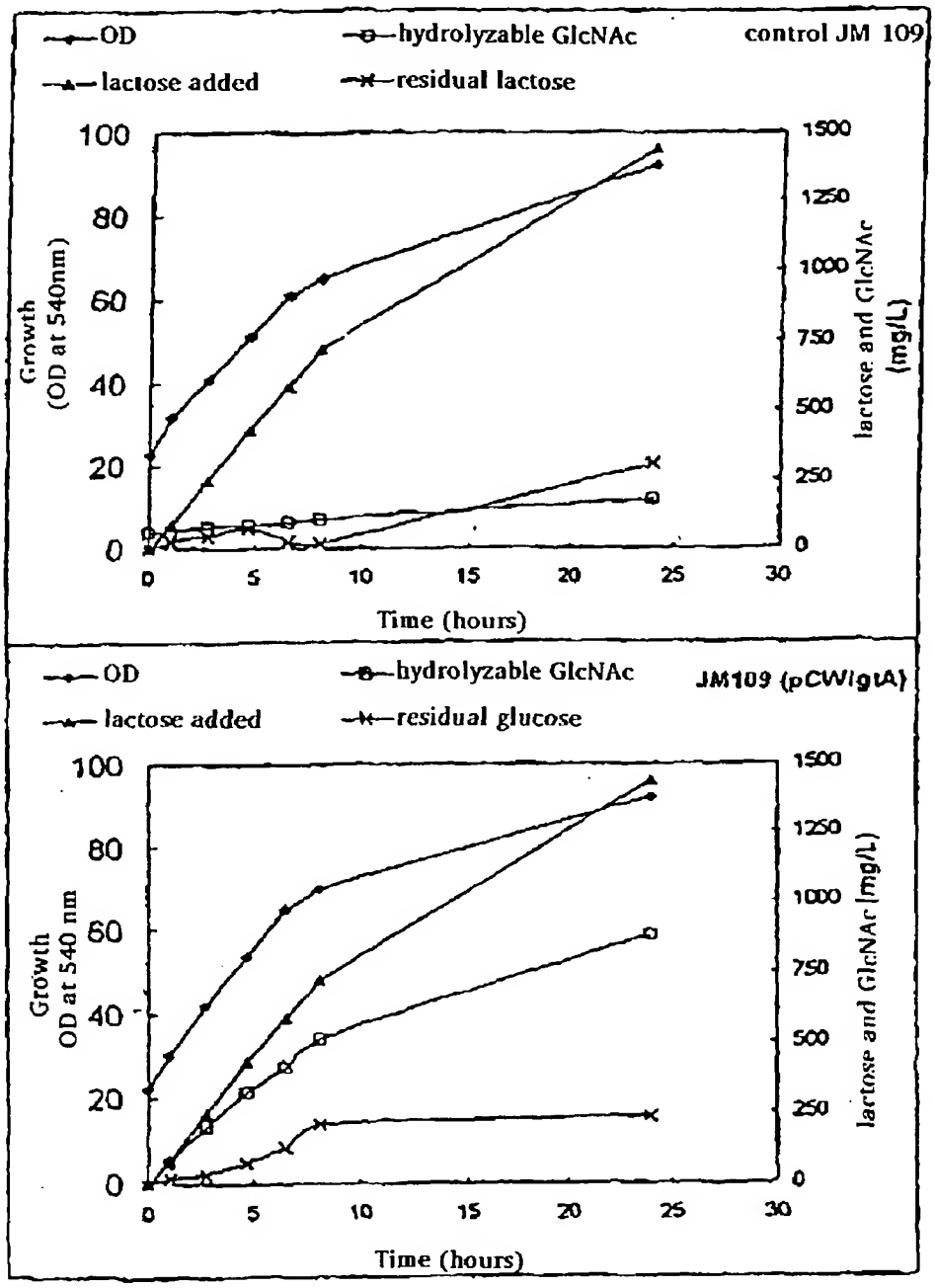
OD
hydrolyzable GlcNAc
control JM 109
lactose added
residual lactose
Growth (OD at 540nm)
lactose and GlcNAc (mg/L)
Time (hours)
JM109 (pCWlgtA)
residual glucose
Growth OD at 540 nm
lactose and GlcNAc (mg/L)

FIG-2

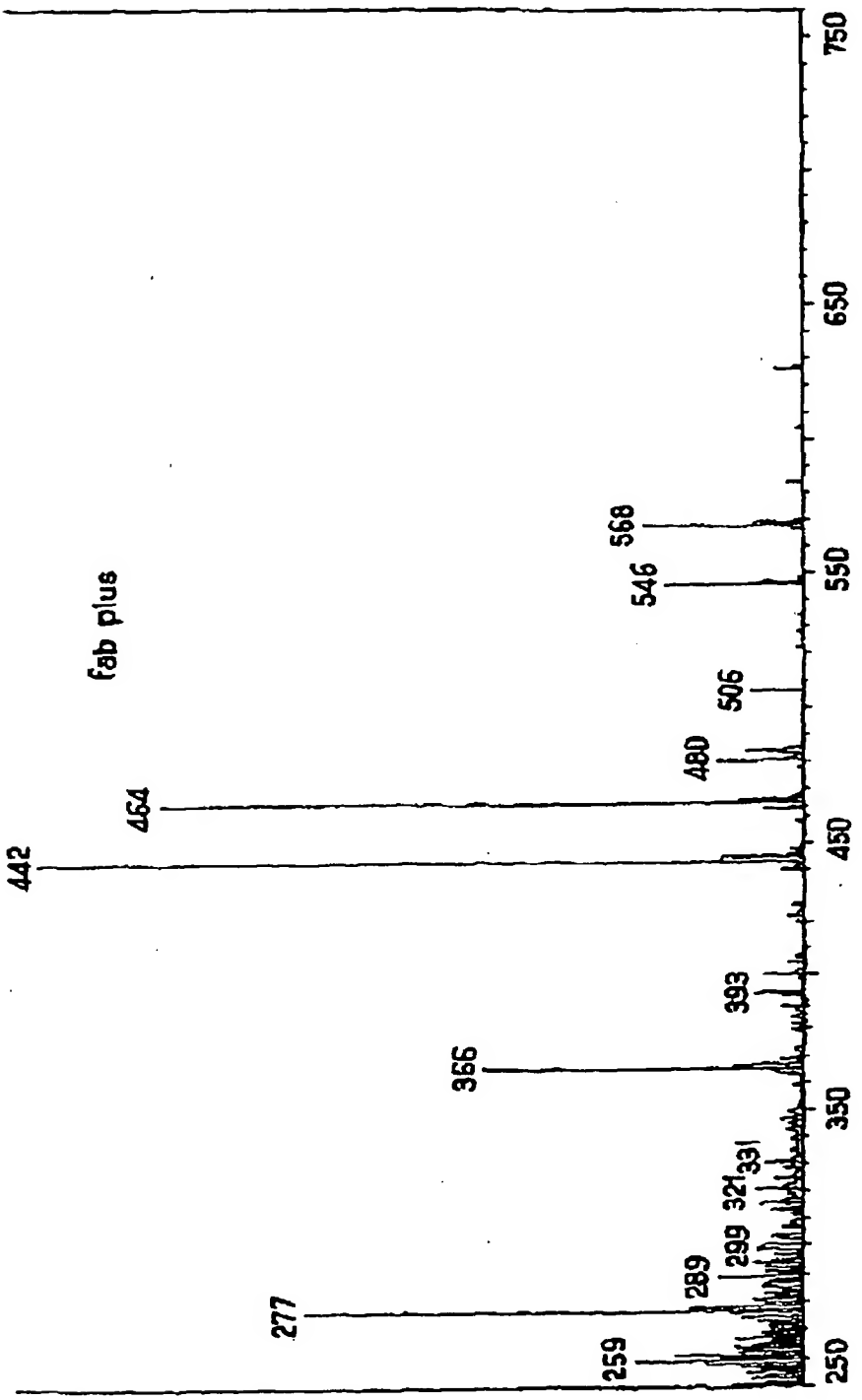
fab plus
750
650
550
450
350
250
568
546
506
480
464
442
393
366
331
321
299
289
277
259

FIG_3

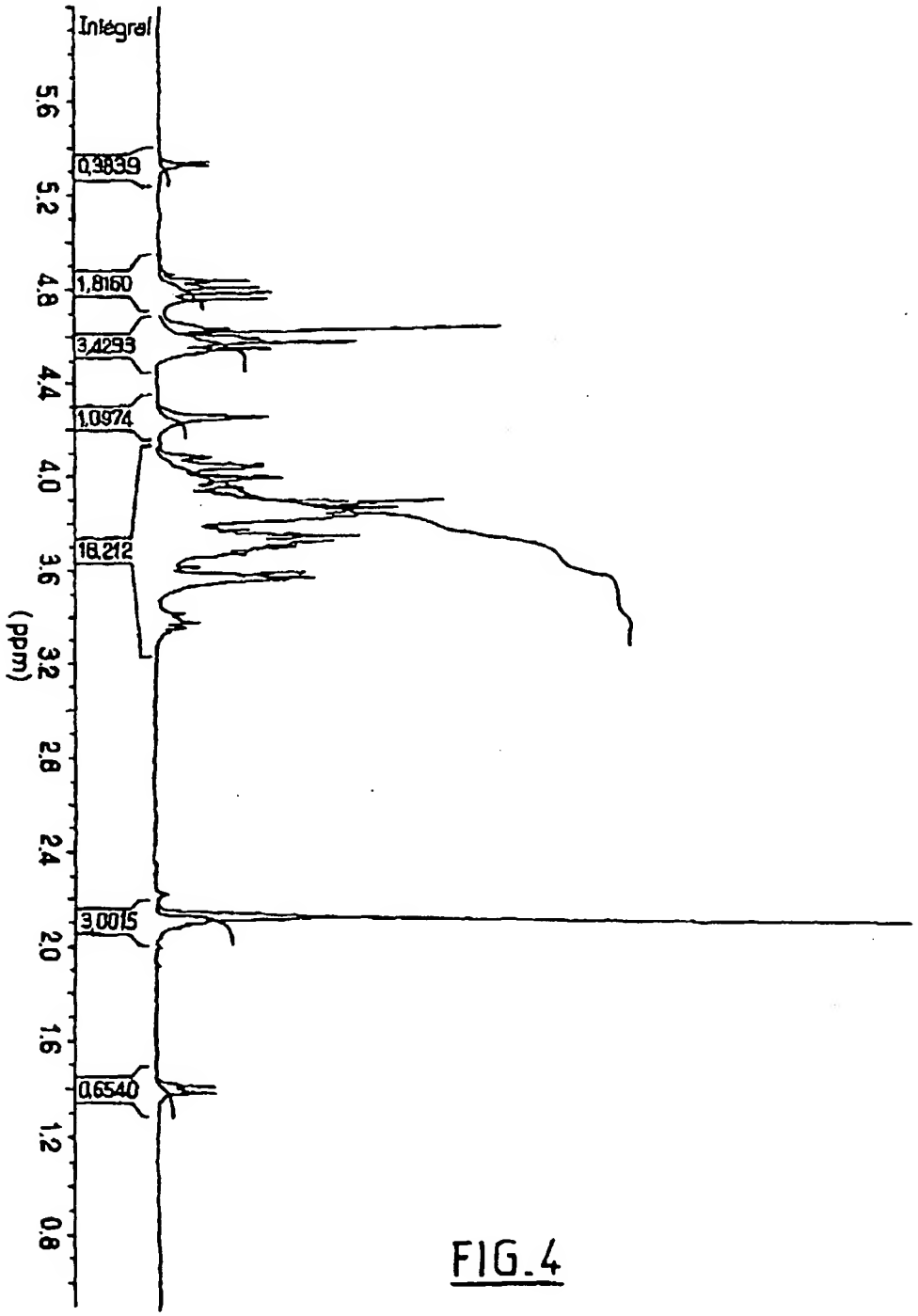
Intégral
0,3839
1,8160
3,4293
1,0974
16,212
3,0015
0,6540
5.6
5.2
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
(ppm)

FIG.4

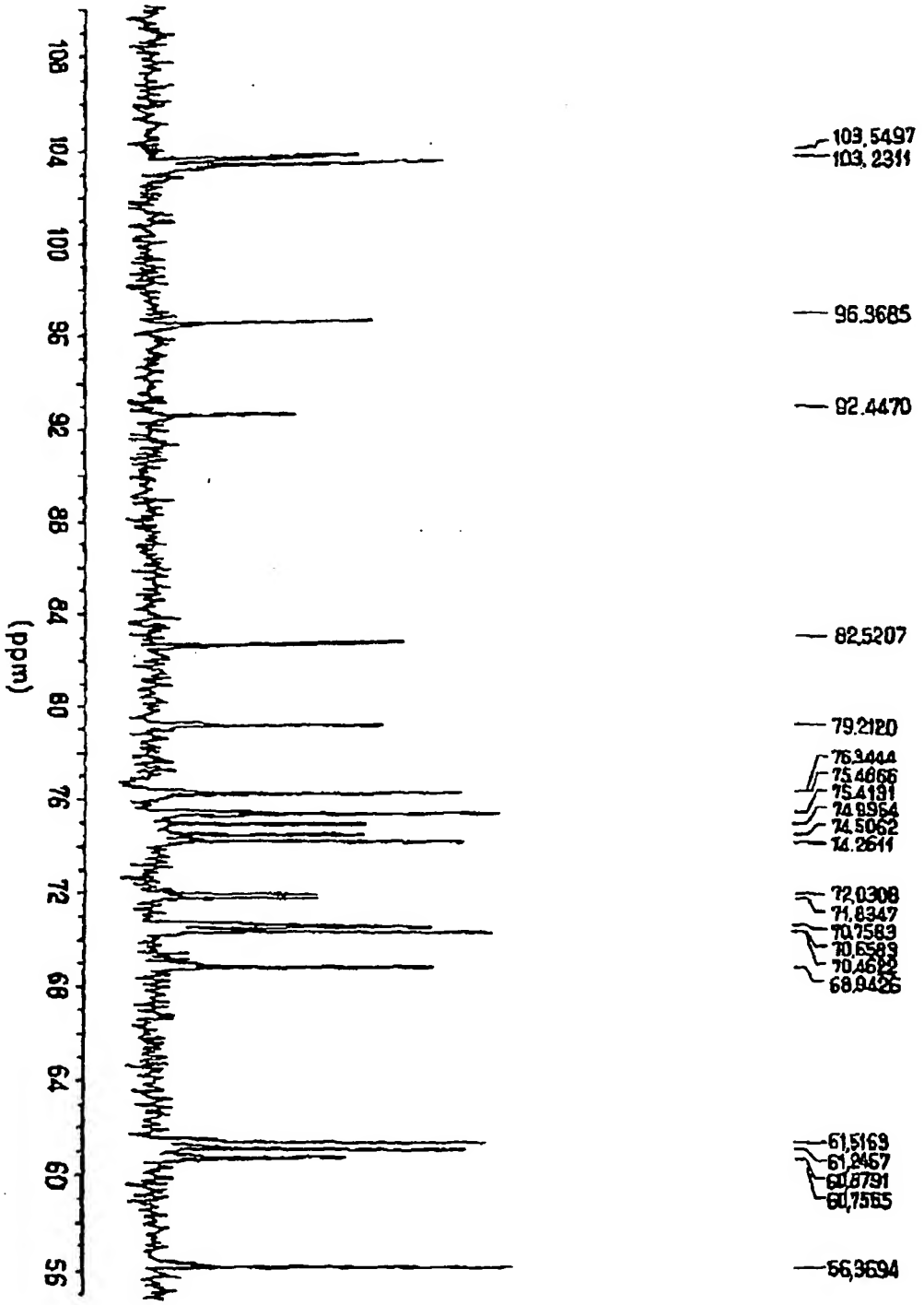
108
104
100
96
92
88
84
80
76
72
68
64
60
56
(ppm)
103.5497
103.2311
96.9685
92.4470
82.5207
79.2120
76.3444
75.4866
75.4131
74.9954
74.5062
74.2611
72.0308
71.8347
70.7583
70.6589
70.4622
68.9426
61.5169
61.2457
60.8791
60.7565
56.3694

FIG_5

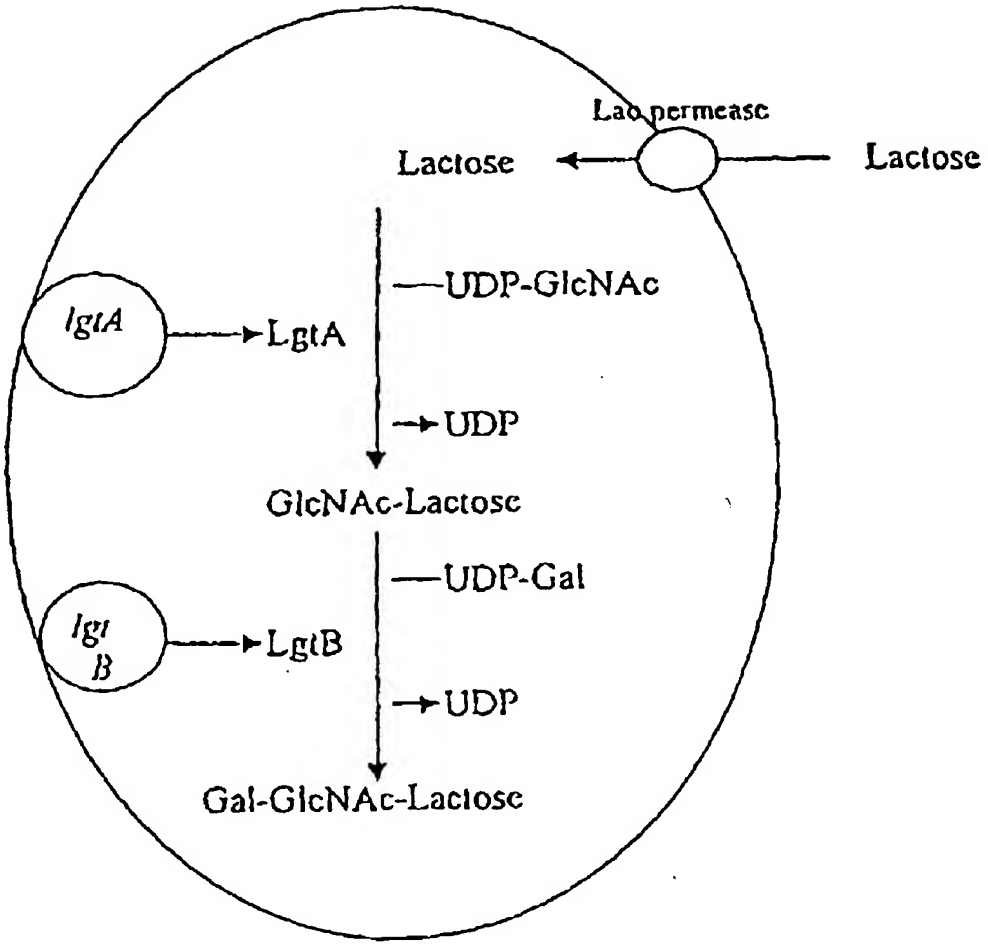
Lac permease
Lactose
Lactose
UDP-GlcNAc
lgtA
LgtA
UDP
GlcNAc-Lactose
UDP-Gal
lgt B
LgtB
UDP
Gal-GlcNAc-Lactose

FIG-6

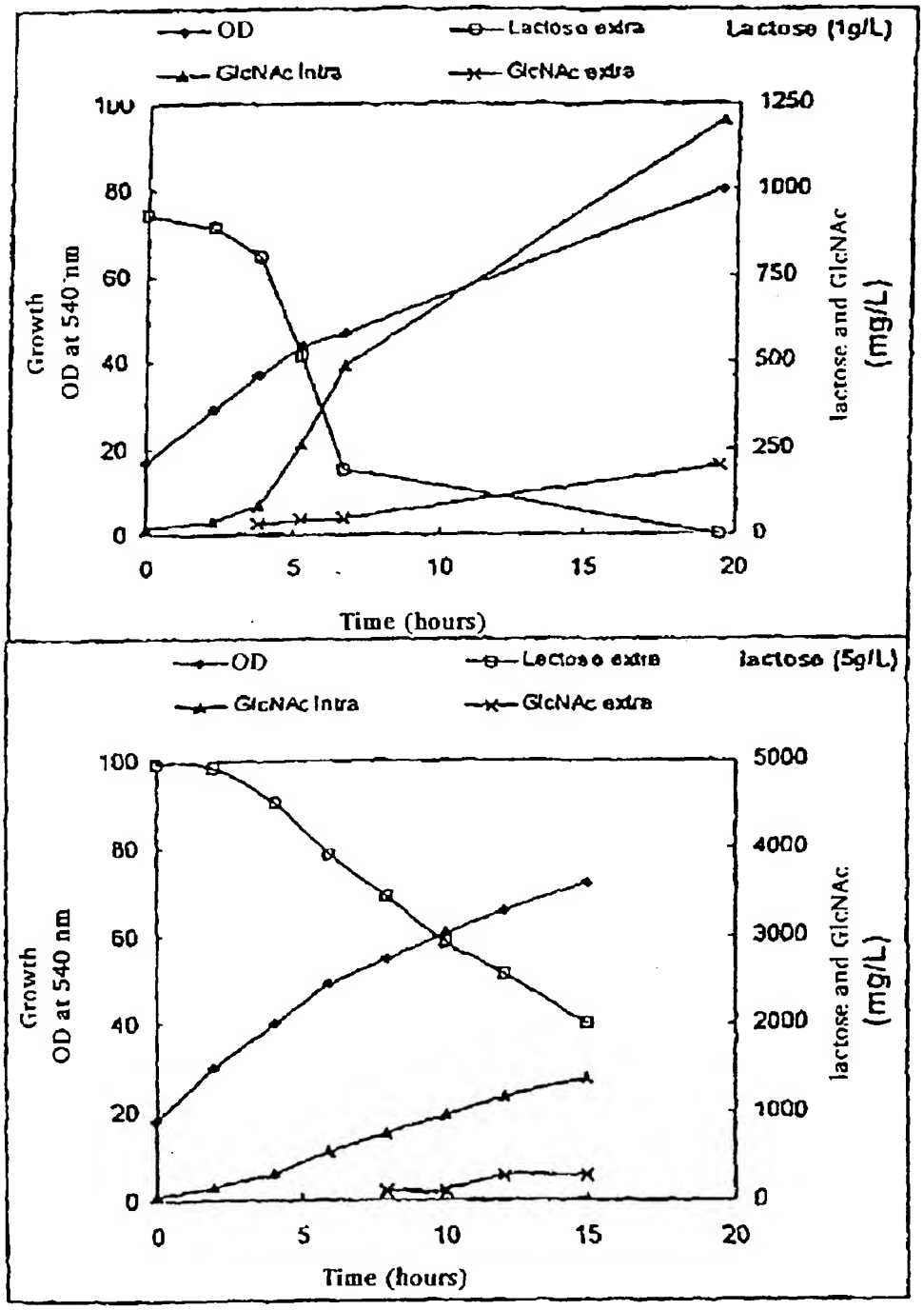
OD
Lactose extra
Lactose (1g/L)
GlcNAc intra
GlcNAc extra
Growth OD at 540 nm
lactose and GlcNAc (mg/L)
Time (hours)
OD
Lactose extra
lactose (5g/L)
GlcNAc intra
GlcNAc extra
Growth OD at 540 nm
lactose and GlcNAc (mg/L)
Time (hours)

FIG-7

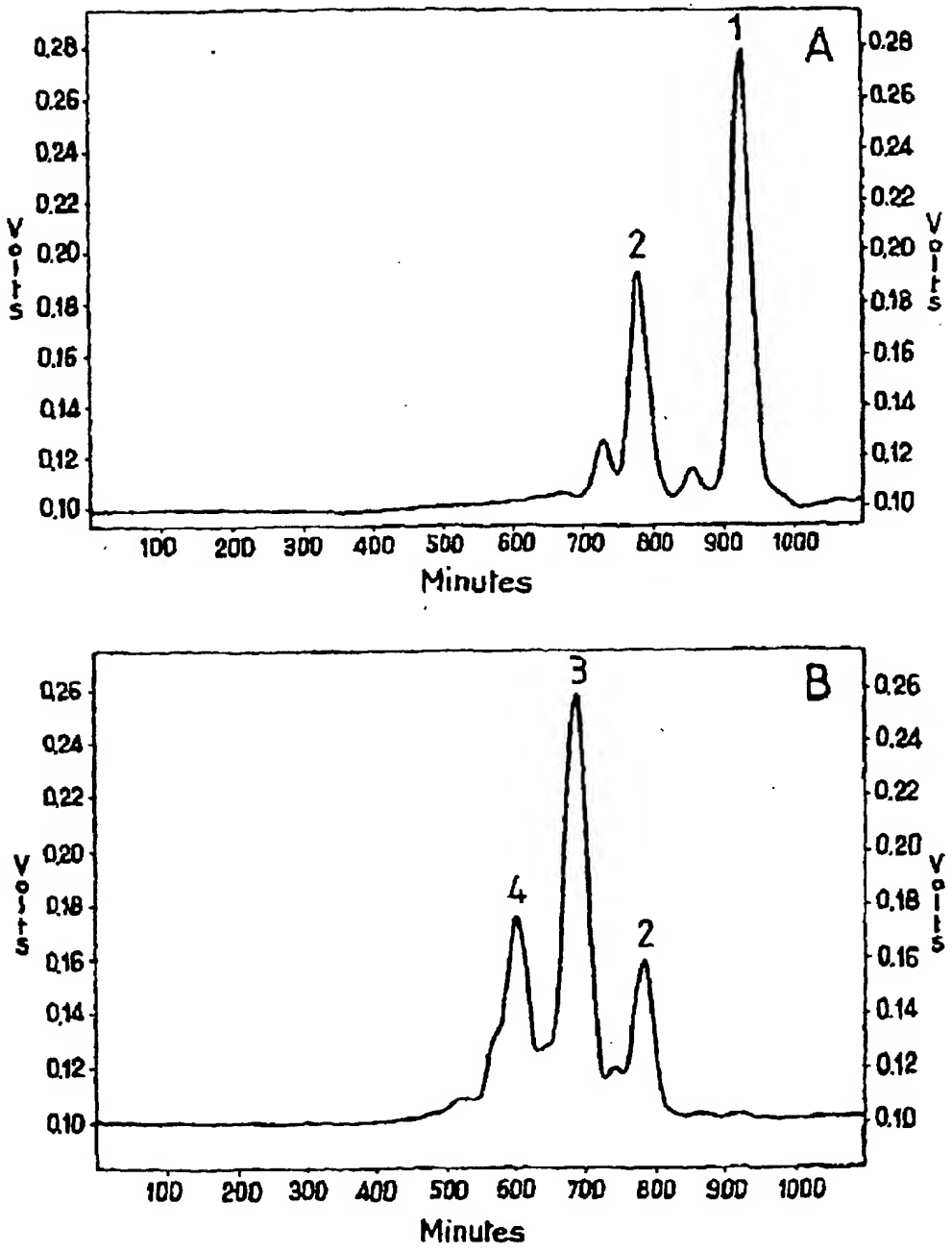
A
1
2
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
Volts
100 200 300 400 500 600 700 800 900 1000
Minutes
B
3
4
2
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
Volts
100 200 300 400 500 600 700 800 900 1000
Minutes

FIG. 8

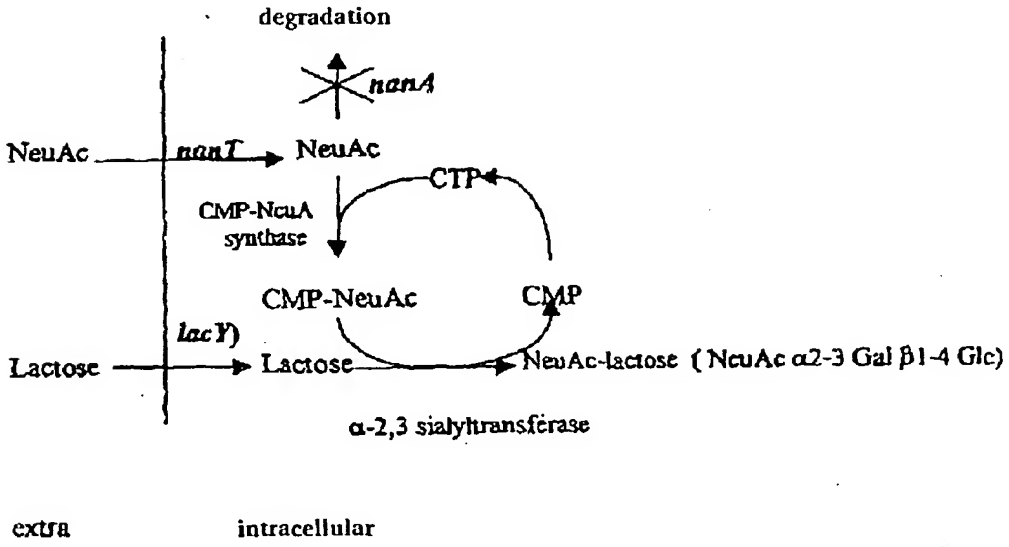
degradation
nanA
NeuAc
nanT
NeuAc
CTP
CMP-NeuA synthase
CMP-NeuAc
CMP
lacY)
Lactose
Lactose
NeuAc-lactose (NeuAc α2-3 Gal β1-4 Glc)
α-2,3 sialyltransferase
extra
intracellular

FIG-9

METHOD FOR PRODUCING OLIGOPOLYSACCHARIDES

The present invention relates to the or even microbiological production of oligosaccharides of biological interest.

It is now well-established that oligosaccharides play an important biological role especially as regards the activity and function of proteins; thus, they serve to modulate the half-life of proteins, and occasionally they are involved in the structure of the protein. oligosaccharides play an essential role in antigen variability (for example blood groups), and in certain bacterial infections such as those caused by *Neisseria meningitidis*.

As oligosaccharides are usually obtained in a low yield by purification starting from natural sources, the synthesis of oligosaccharides has become a major challenge of carbohydrate chemistry, so as to supply sufficient amounts of well-characterized oligosaccharides, required for fundamental research or for any other potential applications (Boons et al., 1996).

The synthesis of complex oligosaccharides of biological interest may be performed chemically, enzymatically or microbiologically.

Despite the development of new chemical methods for synthesizing oligosaccharides in the course of the last 20 years, the chemical synthesis of oligosaccharides remains very difficult on account of the numerous selective protection and deprotection steps, the lability of the glycoside linkages, the difficulties in obtaining regiospecific couplings, and the low production yields. As the number of steps increases with the size of the oligosaccharide, the preparation of large quantities of oligosaccharides longer than trisaccharides is no simple matter. Contrary to the experience of peptide synthesis or nucleic acid synthesis, traditional synthetic organic chemistry cannot at the present time provide a high-quality and large-quantity synthesis of oligosaccharides, even of simple formula.

Consequently, the enzymatic methods have become more popular since they allow a regioselective synthesis under mild conditions and without a step for protection of the hydroxyl groups. The development of the enzymatic approach was made possible by the cloning and functional identification of numerous genes encoding the enzymes involved in the synthetic pathway of oligosaccharides. Thus, various types of enzyme may be used for the in vitro synthesis of oligosaccharides. The physiological function of the glycosyl-hydrosylases and of the glycosyl-phosphorylases is to depolymerize the oligosaccharides, but they may also be used in vitro in the synthesis of oligosaccharides by controlling the reaction equilibrium and kinetics. The substrates of the enzymes for these reactions are readily available, but these enzymatic reactions are not very versatile. Another enzymatic method developed uses the glycosyl-transferases of the Leloir biochemical pathway, which show strong regiospecificity for the precursor and also for the donor substrate; these glycosyl-transferases are not as readily available as the glycosyl-hydrolases. The recombinant DNA technique has recently made it possible to clone and produce a certain number of them. However, the main limitation of this enzymatic method lies in the very high cost of the sugar-nucleotides that are the sugar donors used by these enzymes.

The microbiological route for producing recombinant oligosaccharides in vivo is the most appealing of the synthetic routes since the bacterium is simultaneously responsible for the biosynthesis of the enzymes, the regeneration of the sugar-nucleotides and, finally, the production of the oligosaccharide.

The first descriptions of the microbiological synthesis of oligosaccharides using recombinant bacteria may be considered to a certain extent as the studies which led to the elucidation of the pathways for the biosynthesis of the nodulation factors; these factors are signal molecules secreted by the *rhizobia* to allow recognition by leguminous plants in the nodulation process. Nodulation factors consist of a chito-oligosaccharide backbone bearing various substitutions. The functional identification of the nod genes involved in the biosynthesis of the nodulation factors was partly performed by identifying the oligosaccharides formed in vivo in strains of *Escherichia coli* expressing these various nod genes (Gérémia et al., 1994; Kamst et al., 1995; Spaink et al., 1994; Mergaert et al., 1995). However, the production of oligosaccharides per se was not the aim of these studies; these products were synthesized only in trace amounts and were identified only by means of using radioactive precursors.

On the other hand, it was recently demonstrated in our laboratory (Samain et al., 1997) that the culturing at high cell density of *Escherichia coli* strains containing the nodC (chito-oligosaccharide synthase) gene made it possible to produce large amounts, of greater than 2 g/l, of "recombinant" chito-oligosaccharides.

However, this technique of microbiological synthesis of oligosaccharides remains limited to the production only of chito-oligosaccharides, due to the unique property of nodC (chito-oligosaccharide synthase) of functioning without a precursor; specifically, the other enzymes glycolyze a specific precursor and their activity is thus dependent on the presence of this precursor in the cell. The problem of the precursor is thus the main obstacle blocking the development of the method and its extension to the production of other types of oligosaccharide.

One subject of the present invention is thus a method for producing an oligosaccharide of interest by a genetically modified cell starting with at least one exogenous precursor internalized by said cell, said precursor being involved in the biosynthetic pathway of said oligosaccharide, said method comprising the steps (i) of obtaining a cell that comprises at least one recombinant gene encoding an enzyme capable of modifying said exogenous precursor or one of the intermediates in the biosynthetic pathway of said oligosaccharide from said exogenous precursor necessary for the synthesis of said oligosacchariden from said precursor, and also the components for expressing said gene in said cell, said cell lacking any enzymatic activity liable to degrade said oligosaccharide, said precursor and said intermediates; (ii) of culturing said cell in the presence of at least one said exogenous precursor, under conditions enabling the internalization according to a mechanism of passive and/or active transport of said exogenous precursor by said cell and the production of said oligosaccharide by said cell.

According to one particular embodiment, the present invention relates to a method as described above, characterized in that said cell also comprises at least one gene encoding an enzyme capable of modifying an endogenous precursor involved in the biosynthetic pathway of said oligosaccharide, said enzyme being identical to or different than the enzyme used in the method described above, and also to the components for expressing said gene in said cell and characterized in that said cell lacks any enzymatic activity liable to degrade said precursor.

The term "oligosaccharides" is intended to denote linear or branched polymers with a variable number of residues, linkages and subunits; the number of residues being greater than 1. Oligosaccharides are carbohydrates that become converted on hydrolysis into several monosaccharide molecules; the

monosaccharides being sugars that cannot be converted into a simpler substance by hydrolysis. Monosaccharides are subdivided into trioses, tetroses, pentoses, hexoses and heptoses depending on the number of carbon atoms in their hydrocarbon-based chain, and also into aldoses and ketoses depending on the presence of an aldehyde function or a ketone function in their molecule. Among the monosaccharides that are most frequently encountered, mention may be made of mannose, glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine. The number of chains of stereoisomeric oligosaccharides is extremely large, due to the large number of asymmetric carbons in the hydrocarbon-based chain.

The expression "exogenous precursor" is intended to denote a compound involved in the biosynthetic pathway of the oligosaccharide according to the invention that is internalized by said cell. The expression "endogenous precursor" is intended to denote a compound involved in the biosynthetic pathway of the oligosaccharide according to the invention that is naturally present in said cell.

The expression "genetically modified cell" is intended to denote a microorganism in which at least one alteration of the DNA sequence has been introduced into its genome in order to give said cell a particular phenotype. Such alterations may thus, for example, give the cell the ability not to degrade or not to modify a compound according to the invention, or not to reduce the DNA rearrangement frequency.

The method according to the invention is characterized in that said cell is a cell chosen from bacteria and yeasts. According to one preferred embodiment of the invention, the bacterium is chosen from the group composed of *Escherichia coli, Bacillus subtilis, Campylobacter pylori, Helicobacter pylori, Agrobacterium tumefaciens, Staphylococcus aureus, Thermophilus aquaticus, Azorhizobium caulinodans, Rhizobium leguminosarum, Neisseria gonorrhoeae* and *Neisseria meningitis*. According to one preferred embodiment of the invention, the bacterium is *Escherichia coli*. According to another embodiment of the invention, the cell is a yeast that is preferably *Saccharomyces cerevsae, Saccharomyces pombe* or *Candida albicans*. The cell according to the invention lacks any enzymatic activity liable to degrade said oligosaccharide, said precursor or said metabolic intermediates.

The nucleic acid sequence encoding the enzyme according to the invention is either naturally present in said cell or is introduced into said cell by the recombinant DNA techniques known to those skilled in the art. In the present description, the term "nucleic acid" will be intended to denote a DNA fragment, which is either double-stranded or single-stranded, or products of transcription of said DNAs, and/or an RNA fragment. According to one preferred embodiment, the nucleic acid sequence which is introduced into said cell by the recombinant DNA techniques and which encode an enzyme involved in the biosynthetic pathway of the oligosaccharide of interest is heterologous. The expression "heterologous nucleic acid sequence" is intended to denote a nucleic acid sequence that is not naturally present in the cell according to the invention. The heterologous nucleic acid sequence according to the invention may originate from any animal or plant, eukaryotic or prokaryotic cell type and may originate from viruses.

Among the prokaryotic cells from which the heterologous nucleic acid sequence originates, mention should be made of bacteria and in particular *Escherichia coli, Bacillus subtilis, Campylobacter pylori, Helicobacter pylori, Agrobacterium tumefaciens, Staphylococcus aureus, Thermophilus aquaticus, Azorhizobium caulinodans, Rhizobium leguminosarum, Rhizobium meliloti, Neisseria gonorrhoeae* and *Neisseria meningitis*.

Among the unicellular eukaryotic cells from which the heterologous nucleic acid sequence originates, mention should be made of yeasts and in particular *Saccharomyces cerevisae, Saccharomyces pombe* and *Candida albicans*.

According to one preferred embodiment, the heterologous nucleic acid sequence originates from plant or animal eukaryotic cells. According to an even more preferred embodiment, the heterologous nucleic acid sequence originates from mammalian cells and preferably from human cells.

According to one preferred embodiment of the invention, the cell according to the invention is the bacterium *Escherichia coli* and the nucleic acid sequence introduced into the bacterium and encoding the enzyme according to the invention preferably originates from a bacterium chosen from the group mentioned above.

According to one preferred embodiment of the invention, the nucleic acid sequence encoding the enzyme according to the invention is introduced into said cell in the form of an expression vector. The vector must comprise a promoter, translation start and stop signals, and also regions suitable for regulating transcription. The vector must be able to be maintained stably in the cell over successive generations and can optionally contain particular signals specifying the secretion of the translated enzyme. These various control signals are chosen as a function of the host cell used. To this end, the nucleic acid sequences may be inserted into autonomous replication vectors within the chosen host or into integrative vectors which become integrated into the genome of the chosen host. Such vectors are prepared according to the methods commonly used by those skilled in the art, and the clones resulting therefrom may be introduced into a suitable host cell by standard methods such as, for example, heat shock or electroporation.

The invention is also directed toward the above cells, characterized in that they are transformed by at least one recombinant isolated nucleic acid encoding the enzyme according to the invention or by at least one recombinant vector as defined above.

The method according to the invention is characterized in that said modification made by said enzyme is chosen from glycosylation, sulfatation, acetylation, phosphorylation, succinylation, methylation and addition of an enolpyruvate group, sialylation and fucosylation. More particularly, the method according to the invention is characterized in that said enzyme is an enzyme capable of carrying out a glycosylation, which is chosen from glycosyl-transferases, glycosyl-hydrolases and glycosyl-phosphorylases. According to one preferred embodiment, the enzyme capable of carrying out the glycosylation is a glycosyl-transferase. According to one preferred embodiment, the glycosyl-transferase according to the invention is chosen from β-1,3-N-acetyl-glucosaminyl-transferase, β-1,3-galactosyl-transferase, α-1,3-N-acetyl-galactosaminyl-transferase, β-1,3-glucuronosyl-transferase, β-1,3-N-acetyl-galactosaminyl-transferase, β-1,4-N-acetyl-galactosaminyl-transferase, β-1,4-galactosyl-transferase, α-1,3-galactosyl-transferase, α-1,4-galactosyl-transferase, α-2,3-sialyl-transferase, α-2,6-sialyl-transferase, α-2,8-sialyl-transferase, α-1,3-fucosyl-transferase, α-1,4-fucosyl-transferase and α-1,2-fucosyl-transferase. The glycosyl-transferases used in the present invention are capable of stereospecific conjugation of specific activated saccharide units on a specific acceptor molecule. The activated saccharides generally consist of uridine diphosphate, guanosine diphosphate and cytidine diphosphate saccharide derivatives. Thus, the activated saccharides may be a UDP-saccharide, a GDP-saccharide or a CMP-saccharide.

Certain genes encoding glycosyl-transferases used in the method according to the invention have been described previously; thus, international patent application WO 96/10086 describes the standard oligosaccharide synthesis: in a first step, the various glycosyl-transferases are produced in recombinant bacteria containing the lgtA, lgtB and lgtC genes of *Neisseria gonorrhoeae*, and, after purifying the recombinant enzymes thus produced, the oligosaccharides are synthesized in vitro in the presence of the required precursors and sugar-nucleotides.

According to certain embodiments of the invention, the enzyme capable of performing an acetylation is encoded by the NodL gene of the bacterium *Azorhizobium caulinodans*. According to another embodiment, the enzyme capable of performing a sulfatation is encoded by the NodH gene of the bacterium *Rhizobium meliloti*.

The method according to the invention is characterized in that said cell culturing is preferably performed on a carbon-based substrate; according to one particular embodiment of the invention, said carbon-based substrate is chosen from glycerol and glucose. Other carbon-based substrates may also be used; mention should be made of maltose, starch, cellulose, pectin and chitin. According to another embodiment, the cell culturing is performed on a substrate composed of amino acids and/or protein and/or lipids.

The method according to the invention is characterized in that said culturing step is performed under conditions allowing the production of a culture with a high cell density; this culturing step comprises a first phase of exponential cell growth ensured by said carbon-based substrate, a second phase of cell growth limited by said carbon-based substrate which is added continuously, and finally a third phase of slowed cell growth obtained by continuously adding to the culture an amount of said substrate that is less than the amount of substrate added in step b) so as to increase the content of oligosaccharides produced in the high cell density culture.

The method according to the invention is characterized in that the amount of substrate added continuously to the cell culture during said phase c) is at least 30% less, preferentially 50% and preferably 60% less than the amount of substrate added continuously during said phase b). The method according to the invention is also characterized in that said exogenous precursor is added during phase b).

According to one embodiment of the invention, the method is characterized in that said exogenous precursor is of carbohydrate nature, preferably of oligosaccharide nature.

The novelty and feasibility of the method according to the invention is based on the use of two modes of internalization of the exogenous precursor that do not destroy the integrity of the cell or attack its vital functions. This especially excludes the standard techniques of membrane permeabilization with organic solvents which block growth and energy metabolism. The two possible modes for internalizing the exogenous precursor use a passive or active transport mechanism.

The invention relates firstly to a method that is characterized in that said exogenous precursor is internalized according to a passive transport mechanism. The expression "internalization by passive transport" is intended to denote the passive diffusion of any of the exogenous precursor across the plasma membrane, the molecular flow being oriented from the zones of highest concentration to the zones of lowest concentration so as to tend finally toward a state of equilibrium. The internalization by passive transport consists in using an exogenous precursor that is small enough and hydrophobic enough to diffuse passively across the membrane. A monosaccharide precursor whose anomeric position is blocked with an alkyl substitute constitutes an example of a

precursor that may be internalized in this manner. The present invention thus relates to a method that is characterized in that said exogenous precursor is a monosaccharide whose anomeric carbon is linked to an alkyl group; preferably, said alkyl group is an allyl group. One of the objects of the invention is thus to provide a method for producing oligosaccharides that contain a functionalizable group such as the allyl group and that can consequently be used as precursors for the chemical synthesis of glycoconjugates (neoglycoprotein or neoglycolipids) or glycopolymers. The reason for this is that the double bond of the allyl group is able to be opened by ozonolysis to form an aldehyde and to allow the oligosaccharide to conjugate onto a protein by reductive amination (Roy et al., 1997). Another route is the addition of cysteamine (Lee and Lee, 1974, Roy et al., 1997) to the allylic double bond to form an amine end group which may react, for example, with the carboxylic groups of proteins.

According to one particular embodiment, the method according to the invention concerns the production of (β-D-Gal-[1→4]-β-D-GlcNac-1→O-allyl); the method is characterized in that said cell is a bacterium of $LacZ^-$ genotype, said enzyme is β-1,4-galactosyl-transferase, said substrate is glycerol and said precursor is allyl-N-acetyl-β-D-glucosaminide (β-D-GlcNac-1→O-allyl). Finally, according to another particular embodiment, the method according to the invention is characterized in that the double bond of the allyl group of said (β-D-Gal-[1→4]-β-D-GlcNac-1→O-allyl) is chemically modified by addition, oxidation or ozonolysis reactions.

The present invention also relates to a method that is characterized in that said precursor is internalized according to an active transport mechanism. The expression "internalization by active transport" is intended to denote the ability of cells and preferably of bacteria to selectively admit and concentrate certain exogenous substances or precursors into their cytoplasm. This transport is performed by transporters of protein nature known as permeases, which act as enzymes; permeases are inducible catalysts, that is to say catalysts that are synthesized in the presence of the substrate or the precursor. According to one particular embodiment of the invention, lactose and β-galactosides constitute precursors that are actively transported into the cytoplasm of the bacterium *Escherichia coli* by lactose permease, also known as galactoside permease. The invention thus relates to a method according to the invention that is characterized in that said active transport of said precursor is performed by lactose permease. Lactose permease has fairly broad specificity, which allows it to transport molecules other than lactose.

The reason for this is that it is capable of transporting various natural or synthetic β-galactosides, α-galactosides and sucrose. One of the objects of the invention is thus to provide, according to a preferred embodiment, a method that is characterized in that said precursor is lactose, which constitutes the base moiety for a great many biologically active oligosaccharides. It is also within the scope of the invention to provide a method that is characterized in that said precursor is chosen from the group composed of: (i) natural or synthetic β-galactosides, preferably from 4-O-β-D-galactopyranosyl-D-fructofuranose (lactulose), 3-O-β-D-galactopyranosyl-D-arabinose and allyl-β-D-galactopyranoside, (ii) α-galactosides, preferably melibiose and raffinose, and allyl-α-D-galactopyranoside, (iii) sucrose.

The specificity of lactose permease may even be modified by mutation and allow the transport of other compounds such as maltose and cellobiose. All these compounds may thus be used as precursors for the synthesis of oligosaccharides. It is also within the scope of the invention to use as precursors lactose analogs containing a chemically reactive group for a

subsequent functionalization of the product; preferably, one of these analogs is allyl-β-D-galactopyranoside. It is also within the scope of this invention to use other permeases possibly modified by recombinant DNA techniques to allow the internalization of different types of precursor.

The β-galactosides are normally hydrolyzed in the cytoplasm of the bacterium by the β-galactosidase encoded by the LacZ gene. In order to overcome this problem, a lacZ⁻ bacterial mutant lacking β-galactosidase activity is used when the precursor used is lactose and/or a β-galactoside. One of the objects of the invention is thus also to provide the method according to the invention that is characterized in that said cell lacks enzymatic activity liable to degrade said precursor or said metabolic intermediates.

According to one particular embodiment, the invention relates to a method described above that is characterized in that said precursor is sialic acid. In this case, said active transport of said precursor is performed by NanT permease.

According to another particular embodiment, the invention relates to a method described above that is characterized in that said precursor is sialic acid and lactose. In this case, said active transport of said precursor is performed by lactose permease and NanT permease.

In the method according to the invention, said cell may be lacking in enzymatic activity liable to degrade said precursor(s).

According to one preferred embodiment, the method is characterized in that said cell has a genotype chosen from LacZ⁻ and/or NanA⁻.

According to another aspect of the invention, the method is characterized in that it also comprises the addition of an inducer to said culture medium to induce the expression in said cell of said enzyme and/or of a protein involved in said active transport; according to one preferred embodiment, the method according to the invention is characterized in that said inducer is isopropyl β-D-thiogalactoside (IPTG) and said protein is lactose permease.

The invention makes it possible for the first time to produce complex oligosaccharides in yields of the order of one gram per liter. Depending on its size, the oligosaccharide either accumulates in the bacterial cytoplasm or is secreted into the culture medium. Thus, according to one preferred embodiment, the method according to the invention is used for the production of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranosyl]-D-glucopyranose, (β-D-GlcNac-[1→3]-β-D-Gal-[1→4]-D-Glc); it is characterized in that said cell is a bacterium of LacZ⁻, LacY⁺ genotype, said enzyme is β-1,3-N-acetyl-glucosaminyl-transferase, said substrate is glycerol, said inducer is isopropyl β-D-thiogalactoside (IPTG) and said precursor is lactose.

According to a second preferred embodiment, the method according to the invention is used for the production of lacto-N-neo-tetraose and polylactosamine; it is characterized in that said cell is a bacterium of LacZ⁻, LacY⁺ genotype, said enzymes are β-1,3-N-acetyl-glucosaminyl-transferase and β-1,4-galactosyl-transferase, said substrate is glucose, said inducer is isopropyl-β-D-thiogalactoside (IPTG) and said precursor is lactose.

According to a third preferred embodiment, the method according to the invention is used for the production of allyl 3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside, (β-D-GlcNac-[1→3]-β-D-Gal-1→O-allyl); it is characterized in that said cell is a bacterium of LacZ⁻, LacY⁺ genotype, said enzyme is β-1,3-N-acetyl-glucosaminyl-transferase, said substrate is glycerol, said inducer is isopropyl β-D-thiogalactoside (IPTG) and said precursor is allyl-β-D-galactopyranoside.

According to a fourth preferred embodiment, the method according to the invention is used for the production of analogs of lacto-N-neo-tetraose and of polylactosamines in which the glucose residue is replaced with an allyl group; it is characterized in that said cell is a bacterium of LacZ⁻, LacY⁺ genotype, said enzymes are β-1,3-N-acetyl-glucosaminyl-transferase and β-1,4-galactosyl-transferase, said substrate is glucose, said inducer is isopropyl β-D-thiogalactoside (IPTG) and said precursor is allyl-β-D-galactopyranoside.

According to a fifth preferred embodiment, the method according to the invention is used for the production of allyl-β-D-lactosamine (β-D-Gal-[1→4]β-D-GlcNac-1→O-allyl); it is characterized in that said cell is a bacterium of LacZ⁻, LacY⁺ genotype, said enzyme is β-1,4-galactosyl-transferase, said substrate is glycerol and said precursor is allyl-N-acetyl β-D-glucosaminide (β-D-GlcNac-[1→O-allyl]).

The invention also relates to a method that makes it possible to envisage the production of a large number of different oligosaccharides obtained by glycosylation of lactose. Specifically, besides the genes lgtA and lgtB which respectively encode β-1,3-N-acetyl-glucosaminyl-transferase and β-1,4-galactosyl-transferase, several genes of bacterial glycosyl-transferases using lactose as precursor have recently been cloned. These are lgtC (β-1,4-galactosyl-transferase) and Lst (α-2,3-sialyl-transferase) (Gilbert et al., 1997). Using these genes in a method according to the invention makes it possible to produce molecules such as globotriose (p^k blood antigen) and sialyl-lactose. Moreover coexpression of the lgtA and lgtB genes with the gene for α-1,3-fucosyl-transferase from *Helicobacter pylori* (Martin et al., 1997) according to a method according to the invention makes it possible to obtain Lewis^x pentasaccharide. The addition of the Lst (α-2,3-sialyl-transferase) gene gives access to the sialyl Lewis^x hexasaccharide.

The method according to the invention also makes it possible to obtain a large number of different oligosaccharides obtained by glycosylation of exogenous precursors other than lactose and transported by lactose permease or by other permeases.

The method according to the invention makes it possible to obtain a large number of different oligosaccharides obtained by in vivo modification (sulfatation, acetylation, phosphorylation, succinylation, methylation, addition of an enolpyruvate group) of precursors. The synthesis of certain oligosaccharides may necessitate the modification of endogenous precursors, in addition to the modification of exogenous precursors. Thus, it may be envisaged to introduce into a K12 *Escherichia coli* bacterium the gene for the enzyme involved in the metabolism of an endogenous precursor to allow the production of certain sugar-nucleotides such as, for example, CMP-sialic acid, UDP-GalNAc or GDP-fucose, which are not normally produced by this bacterial strain, so as to achieve the synthesis of an oligosaccharide of interest. For example, UDP-GalNAc may be produced from UDP-GlcNAc if the epimerase gene is introduced into a cell according to the invention.

Contrary to the enzymatic method for the in vitro synthesis of oligosaccharides, which requires the use of very expensive molecules such as ATP, acetyl-CoA, PAPS (adenosine 3'-phosphate 5'-phosphosulfate) or phospho-enolpyruvate, one of the advantages of the present invention lies in the fact that these molecules are naturally recycled into the cell, thus making it possible to reduce the production costs of the oligosaccharides.

Another subject of the invention relates to a method described above for the production of 3'-sialyllactose (α-NeuAc-[2→3]-→-D-Gal-[1→4]-β-D-Glc) or 6'-sialyllactose (α-NeuAc-[2→6]-β-D-Gal-[1→4]-β-D-Glc), characterized in that:

said cell is a bacterium of LacZ$^-$, LacY$^+$, NanA$^-$ or NanI$^+$ genotype;

said enzymes are CMP-NeuAc-synthase and α-2,3-sialyltransferase or α-2,6-sialyl-transferase;

said substrate is glycerol;

said inducer is isopropyl-β-D-thiogalactoside (IPTG);

said precursors are lactose and sialic acid.

According to a sixth preferred embodiment which completes the second embodiment described above, the method according to the invention is. used for the production of a sialyl derivative of lacto-N-neo-tetraose and of polylactosamine (lacto-N-neo-hexaose, lacto-N-neo-octaose, lacto-N-neo-decaose), characterized in that it also comprises a said enzyme chosen from α-2,3-sialyl-transferase and α-2,6-sialyl-transferase, and in that said cell also has a NanA$^-$, NanI$^+$ genotype and expresses the CMP-NeuAc-synthase gene, said acceptors are lactose and sialic acid.

Another subject of the invention relates to a method described above for the production of lacto-N-neo-tetraose, β-D-Gal[1→4]-β-D-GlcNAc[1→3]-β-D-Gal[1→4]-(α-L-Fuc-[1→3])-β-D-Glc, β-D-Gal[1→4]-(α-L-Fuc-[1→3])-β-D-GlcNAc[1→3]-β-D-Gal [1→4]-(α-L-Fuc-[1→3])-β-D-Glc, β-D-Gal[1→4]-(α-L-Fuc-[1→3])-β-D-GlcNAc [1→3]-D-Gal [1-4]-β-D-GlcNAc-[143]-β-D-Gal[1→4]-(α-L-Fuc-[1→3])-β-D-Glc, characterized in that

said cell is a bacterium of LacZ$^-$, LacY$^+$, WcaJ$^-$ genotype and overexpresses rcsA;

said enzymes are β-1,3-N-acetyl-glucosaminyl-transferase, β-1,4-galactosyl-transferase and α-1,3-fucosyl-transferase;

said substrate is glucose;

said inducer is isopropyl-β-D-thiogalactoside (IPTG);

said precursor is lactose.

According to a seventh preferred embodiment, the method according to the invention is used for the production of 3'-fucosyllactose (β-D-Gal-[1→4]-(α-L-Fuc-[1→3]-D-Glc) or 2'-fucosyllactose (β-D-Gal-[1→2]-(α-L-Fuc-[1→3]-D-Glc), characterized in that it comprises a said enzyme chosen from α-1,3-fucosyl-transferase or α-1,2-fucosyl-transferase, and in that the cell has a wcaj$^-$ lacZ$^-$ genotype and overexpresses the rcsA gene and in that said precursor is lactose.

According to an eighth preferred embodiment, the method according to the invention is used for the production of a fucosyl derivative of lacto-N-neo-tetraose and of polylactosamine (lacto-N-neo-hexaose, lacto-N-neo-octaose, lacto-N-neo-decaose), characterized in that it also comprises a said enzyme chosen from α-1,2-fucosyl-transferase and α-1,3-fucosyl-transferase, and in that said cell also has a WcaJ$^-$ genotype and overexpresses the RcsA gene, said acceptor being lactose.

According to a ninth preferred embodiment, the method according to the invention is used for the production of a sialyl and fucosyl derivative of lacto-N-neo-tetraose, lacto-N-neo-decaose), characterized in that it also comprises a said enzyme chosen from α-2,3-sialyl-transferase and α-2,6-sialyl-transferase, and also a said enzyme chosen from α-1,2-fucosyl-transferase and α-1,3-fucosyl-transferase, and in that said cell also has a NanA$^-$, NanI$^+$, WcaJ$^-$ genotype and overexpresses the RcsA gene and the gene for CMP-NeuAc-synthase, said acceptors are lactose and sialic acid.

The methods of embodiments 1 to 9 mentioned above may be carried out for the production of oligosaccharide analogs

in which the glucose residue is replaced with an allyl group, said precursor now being allyl-β-D-galactoside rather than lactose.

Another object of the invention is to provide a method for producing oligosaccharides that are labeled with or enriched in radioisotopes; such oligosaccharides are extremely precious for fundamental biological or conformational analysis studies. The invention thus relates to a method for producing an oligosaccharide that is labeled with at least one radioisotope, characterized in that said cell is cultured on said carbon-based substrate labeled with said radioisotope and/or in the presence of a said precursor labeled with said radioisotope. The radioisotopes are preferably chosen from the group composed of: ^{14}C, ^{13}C, ^{3}H, ^{35}S, ^{32}P, ^{33}P.

The invention also relates to an oligosaccharide which may be obtained by a method according to the invention.

According to one particular embodiment, the invention relates to an activated oligosaccharide that may be used for the chemical synthesis of glycoconjugates or glycopolymers that may be obtained by a method as described above, said oligosaccharide being characterized in that the double bond of the allyl group is chemically modified by addition, oxidation or ozonolysis reactions.

The oligosaccharide according to the invention is useful in a wide range of therapeutic and diagnostic applications; it may be used, for example, as an agent for blocking cell surface receptors in the treatment of a host of diseases involving cellular adhesion, or may be used as nutritional supplements, antibacterial agents, anti-metastatic agents and anti-inflammatory agents. The invention thus relates to an oligosaccharide according to the invention as a medicinal product, and especially as a medicinal product intended for selectively preventing the adhesion of biological molecules. The oligosaccharide according to the invention is also used as a medicinal product intended for treating cancer, inflammation, heart diseases, diabetes, bacterial infections, viral infections and neurological diseases and as a medicinal product intended for grafts. The invention also relates to a pharmaceutical composition, characterized in that it comprises an oligosaccharide according to the invention and a pharmaceutically acceptable vehicle.

Finally, the invention also relates to the agricultural and agronomic use of an oligosaccharide according to the invention, especially for the growth and defense of plants. Specifically, oligosaccharides play a predominant role in *Rhizobium*/leguminous plant symbiosis. Indeed, certain oligosaccharides originating from the hydrolysis of fungal or plant glycoproteins or walls can act as plant hormones or as elicitors of defense reactions in plants.

The industrial advantage of the method according to the invention is obvious since it makes it possible for the first time to achieve a production of the order of a kilogram of complex oligosaccharides of biological interest. All the oligosaccharides of biological interest that we envisage synthesizing at the industrial scale are currently available only at the mg scale and at extremely high costs (up to 1 million FFr per gram); the cost price of these compounds produced by the present microbiological route are infinitely lower.

The characteristics and advantages of the present invention will be demonstrated more clearly on reading the examples and figures which follow, the keys to which are represented below.

FIGURES

FIG. 1: Principle of the method for producing the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy-β-D-gluco-pyra-

nosyl)-β-D-galactopyranosyl]-D-glucopyranose, (β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-D-Glc)

Lactose (β-D-Gal-[1-4]-β-D-Glc) is transported into the cell by lactose permease (Lac permease). The lactose cannot be hydrolyzed in the cell since the strain is a LacZ⁻ mutant. Expression of the lgtA gene allows the production of the LgtA enzyme which transfers a GlcNAc from UDP-GlcNAc onto a lactose molecule. The trisaccharide formed (β-D-GlcNAc-[1-3]-β-D-Gal-[1-4]-D-Glc) is excreted into the medium.

FIG. 2: High cell density culturing of the control strain JM 109 and of the strain JM 109 (pCWlgtA) containing the glycosyl transferase gene LgtA

Lactose is added continuously and the residual lactose is determined enzymatically. The concentration of hydrolyzable GlcNAc in the culture medium is measured calorimetrically after acid hydrolysis. The added lactose represents the total cumulative amount of lactose which was added continuously.

FIG. 3: Mass spectrum in FAB⁺ mode of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy-β-D-gluco-pyranosyl)-β-D-galactopyranosyl]-D-glucopyranose, (β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-D-Glc) purified from the culture supernatant of the strain JM 109 (lgtA)

The two quasi-molecular ions $[M+H]^+$ and $[M+Na]^+$ are observed at m/z 546 and 568. An ion $[M+H]^+$ at m/z 442 is also observed, which is due to the presence of β-D-GlcNAc-[1-3]-IPTG. This indicates that the IPTG (isopropyl β-D-thiogalactose) used to induce Lac permease and LgtA is also glycosylated.

FIG. 4: Proton NMR spectrum of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranosyl]-D-glucopyranose, (β-D-GlcNAc-[1→3-]1-β-D-Gal-[1→4]-D-Glc) at 323° K

The signal at 1.4 ppm is due to the protons of the isopropyl group of the glycosylated IPTG derivative.

FIG. 5: ^{13}C NMR spectrum of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galacto-pyranosyl]-D-glucopyranose, (β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-D-Glc)

FIG. 6: Principle of the method for producing lacto-N-neo-tetraose (β-D-Gal-[1-4]-β-D-GlcNAc-[1-3]-β-D-Gal-[1-4]-β-D-Glc)

Lactose (β-D-Gal-[1-4]-β-D-Glc) is transported into the cell by Lac permease. The lactose cannot be hydrolyzed in the cell since the strain is a LacZ⁻ mutant. Expression of the lgtA gene allows the production of the LgtA enzyme which transfers a GlcNAc from UDP-GlcNAc onto a lactose molecule. The trisaccharide formed is then used as a precursor by LgtB which transfers a galactose molecule from UDP-Gal to form lacto-N-neo-tetraose (β-D-Gal-[1-4]-β-D-GlcNAc-[1-3]-β-D-Gal-[1-4]-β-D-Glc).

FIG. 7: High cell density culturing of the strain JM 109 (pCWlgtA, pBBlgtB)

Culturing in the presence of lactose at high concentration (5 $g.l^{-1}$) and at low concentration (1 $g.l^{-1}$).

FIG. 8: Separation on Biogel P4 of the oligosaccharides produced by the strain JM 109 (pCWlgtA, pBBlgtB) in the presence of lactose at an initial concentration of 5 $g.l^{-1}$ (A) or of 1 $g.l^{-1}$ (B)

The peaks 1, 2, 3 and 4 correspond, respectively, to lacto-N-neo-tetraose, lacto-N-neo-hexaose, lacto-N-neo-octaose and lacto-N-neo-decaose.

FIG. 9: Principle of the method for producing sialyllactose

Lactose and sialic acid (NeuAc) are internalized in the cell by lactose permease (lacy) and sialic acid permease (nanT). These two compounds are not degraded in the cell since the strain is a lacZ⁻ and nanA⁻ mutant. The expression of CMP-NeuA synthase and of α-2,3-sialyl-transferase allows the activation of the sialic acid internalized into CMP-NeuAc and its transfer onto the intracellular lactose.

EXAMPLES

Example 1

Materials and Methods

1.1. Origin of the Plasmids and Bacterial Strains

The strains JM 107 and JM 109 of *Escherichia coli* K12 (Yannisch-Perron et al., 1984) were used as host cells for all the oligosaccharide production examples described. The strains were obtained from the DSM (Deutsche Sammlung von Mikroorganismen). The genotype of the strain JM 109 is as follows: F traD36 $lacI^q$ Δ(lacZ)M15 $proA^+B^+/e14^-$ ($McrA^-$) Δ(lac-proAB) supE44 recA1 endA1 gyrA96 (Nal^r) thi hsdR17 relA1. The genotype of the strain JM 107 is identical to that of the strain JM 109 except for the fact that the recA1 gene is not inactivated.

The lgtA and lgtB genes of *Neisseria* meningitis MC58 were supplied by Dr W. Wakarchuk (Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario, K1A OR6, Canada) in the form of two plasmids pCW, one containing the lgtA gene (referred to herein as pCWlgtA) and the other containing the lgtB gene (referred to herein as pCWlgtB). The sequences of these two genes are available from the GenBank databank under the number U25839. The plasmid pLitmus28 was purchased from the company New Englands Biolabs. The plasmid pBBR1MCS was supplied by Dr M. Kovach (Department of Microbiology and Immunology, Louisiana State University, Shreveport, La. 71130-3932, USA).

The genes for CMP-sialic acid synthase and α-2,3-sialyl-transferase of *Neisseria meningitis* MC58 were supplied by Dr M. Gilbert (Institute for Biological Sciences, National Research Council of Canada, '100 Sussex Drive, Ottawa, Ontario, K1A OR6, Canada) in the form of two plasmids NSY-01 and NST-01. The plasmid NSY-01 is a derivative of the plasmid pT7-7 which contains the gene (GenBank U60146) for CMP-sialic acid synthase (Gilbert et al., 1997). The plasmid NST-01 is a derivative of the plasmid pBluescript Sk^- which contains the gene (GenBank No. U60660) for α-2,3-sialyl-transferase (Gilbert et al., 1996).

The gene fucT for α-1,3-fucosyl-transferase of *Helicobacter pylori* was supplied by Dr S. Martin (Glaxo Wellcome Research and Development, Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY, UK) in the form of a plasmid pHP0651 derived from pET-21a. The sequence is available from the GenBank (AE000578, gene HPO651).

1.2. Subclonings

We used the standard techniques of molecular biology described by Sambrook et al. (1989).

construction of the plasmid pBBlgtB: the 0.835-kb DNA fragment containing the lgtB gene was obtained by digestion of the plasmid pCWlgtB with BamHI and HindIII. This fragment was subcloned into the vector pLitmus28 predigested with BamHI and HindIII to form the plasmid pLitlgtB. The 0.9-kb fragment containing the lgtB gene was excised from the plasmid pLitlgtB by digestion with XhoI and HindIII and subcloned into the plasmid pBBR1MCS predigested with XhoI and HindIII to form the plasmid pBBlgtB.

construction of the plasmid pBBnsy: The fragment containing the gene for CMP-sialic acid synthase was excised from the plasmid NSY-01 by digestion with XbaI 10 and

subcloned into the plasmid pBBRIMCS predigested with XbaI to form the plasmid pBBnsy.

construction of the plasmid pBBLnt: the lgtA gene present in the construct pCWlgtA (Gilbert et al.) was amplified by PCR at the same time as the UV5 tactac promoter of the plasmid using the primers CTTTAAGCTTCCGGCTCG-TATAA (SEQ ID NO: 1) (sense, upstream promoter) and GACAGCTTATCATCGATAAGCTT (SEQ ID NO: 2) (anti-sense, lgtA end) both containing a HindIII site. The 1.3-kb amplified fragment was then subcloned into the HindIII site of the vector pBBlgtB.

construction of the plasmid pBBLntRcsA: The rcsA gene (Stout et al., 1991) was first amplified by PCR starting with genomic DNA from JM 109 with the primers AGGGTAC-CCATGTTGTTCCGTTTAG (SEQ ID NO: 3) (KpnI site, rcsA left) and AATCTAGAGTAATCTTATTCAGCCTG (SEQ ID NO: 4) (XbaI site, rcsA right), and then cloned into the KnpI-XbaI sites of the vector pBBR1-MCS. The vector pBBR1-MCS-rcsA was then opened upstream of the gene by digestion with KpnI, rendered blunt (Amersham kit), freed with XbaI and inserted into the SmaI-XbaI sites of the construct pBBLnt, allowing a cloning downstream of the lgtB-UV5tactac-lgtA assembly, placing rcsA under the control of the UV5 tactac promoter.

1.3. Culture Conditions

The routine cultures and the preparation of the inocula were performed on LB medium (Sambrook et al., 1989). The high cell density cultures were prepared in a 2-liter fermenter containing an initial volume of 1 liter of medium having the following composition: glycerol (17.5 $g.l^{-1}$) or glucose (15 $g.l^{-1}$), $NH_4H_2PO_4$ (7 $g.l^{-1}$), KH_2PO_4 (7 $g.l^{-1}$), $MgSO_4.7H_2O$ (1 $g.l^{-1}$), thiamine HCl (4.5 $mg.l^{-1}$), solution of trace elements (7.5 $ml.l^{-1}$), citric acid (0.5 $g.l^{-1}$), KOH (2 $g.l^{-1}$). The $MgSO_4$ is autoclaved separately and the thiamine is sterilized by filtration. The solution of trace elements contains: nitrilo-triacetate (70 mM, pH 6.5), ferric citrate (7.5 $g.l^{-1}$), $MnCl_2.4H_2O$ (1.3 $g.l^{-1}$), $CoCl_2.6H_2O$ (0.21 $g.l^{-1}$), $CuCl_2.2H_2O$ (0.13 $g.l^{-1}$), H_3BO_3 (0.25 $g.l^{-1}$), $ZnSO_4.7H_2O$ (1.2 $g.l^{-1}$), $Na_2MoO_4.2H_2O$ (0.15 $g.l^{-1}$). The antibiotics ampicillin (50 $mg.l^{-1}$) and chloramphenicol (25 $mg.l^{-1}$) are added to ensure the presence of the various plasmids. The feed solution contains glycerol (500 $g.l^{-1}$) or glucose (400 $g.l^{-1}$), $MgSO_4.7H_2O$ (12 $g.l^{-1}$) and the solution of trace elements (25 $ml.l^{-1}$).

The high cell density cultures are inoculated at 2%.

Throughout the culturing, the dissolved oxygen content is maintained at 20% of saturation by manually controlling the flow rate of air and by automatically adjusting, the stirring speed. The pH is automatically maintained at 6.8 by addition of aqueous ammonia (15% w/v). The temperature is maintained at 34° C. for the strain JM 109(pCWlgtA) and at 28° C. for the strain JM 109(pCWlgtA, pBBlgtB). The high-density culture strategy generally comprises three phases: a first phase of exponential growth which is ensured by the carbon-based substrate (glycerol or glucose) initially present in the medium; a second phase which starts when the growth becomes limited by the carbon source, which is then added continuously at a rate of 4.5 $g.h^{-1}.l^{-1}$ of glycerol or 3.6 $g.h^{-1}.l^{-1}$ of glucose. In a third phase, this rate is reduced by 60% to slow down the growth, so as to increase the oligosaccharide content.

1.4. Assay of the Oligosaccharides

Samples (1 ml) are taken during the culturing and are immediately centrifuged in microtubes. The supernatant is retained to assay the extracellular oligosaccharides. The bacterial pellet is resuspended in 1 ml of water and is then incubated in a water bath at 100° C. for 30 minutes to rupture the cells. After a second centrifugation, the supernatant is retained to assay the intracellular oligosaccharides.

The lactose concentration is measured using an enzymatic determination kit (Roche diagnostic). The N-acetyl-glucosamine residues present in the oligosaccharides are freed by acid hydrolysis as described previously (Samain et al., 1997) and then quantified calorimetrically by the method of Reissig et al., (1955); in the description, the term "hydrolyzable GlcNAc" means the amount of GlcNAc assayed in this way.

Assaying the lactose with and without treatment with a neuraminidase makes it possible to estimate the sialyl-lactose concentration.

The total fucose is measured calorimetrically by the cysteine hydrochloride method of Dische and Shettles (1948).

1.5. Purification of the Oligosaccharides

At the end of the culturing, the bacterial cells are harvested by centrifugation. The supernatant is retained for purification of the extracellular oligosaccharides. The bacterial cells are resuspended in 1 liter of water and are then permeabilized by means of a heat treatment (30 minutes at 100° C.) to release the intracellular oligosaccharides. After a second centrifugation, these oligosaccharides are recovered in the supernatant.

The first and the second supernatant containing the extracellular and intracellular oligosaccharides, respectively, are adsorbed onto active charcoal (100 g per liter of supernatant). After rinsing with distilled water, the oligosaccharides are diluted with 50% (v/v) ethanol, concentrated by evaporation and freeze-dried.

The oligosaccharides are separated out by steric exclusion chromatography on a column (4.5 cmx95 cm) of Biogel P4, allowing the injection of about 300 mg of oligosaccharide mixture. The elution is performed with distilled water, at a flow rate of 40 $ml.h^{-1}$.

The nonfucosyl oligosaccharides are separated out by steric exclusion chromatography on a column (4.5 cmx95 cm) of Biogel P4, allowing the injection of about 300 mg of oligosaccharide mixture. The elution is performed with distilled water at a flow rate of 40 $ml.h^{-1}$.

The fucosyl oligosaccharides are separated out by steric exclusion chromatography on a column (1.5 cmx200 cm) of Biogel P2 thermostatically maintained at 60° C., allowing the injection of about 30 mg of oligosaccharide mixture. The elution is performed with distilled water at a flow rate of 30 $ml.h^{-1}$.

The sialyllactose is separated from the neutral oligosaccharides by binding onto a Dowex 1X4-400 resin (in HCO_3^- form), and eluted with a gradient of $NaHCO_3$ (0 to 100 mM). The bicarbonate is then removed by treating the eluate with a Dowex 50X4-400 resin in in H^+ form.

1.6. Preparation of the Allyl β-D-Glucosides

Allyl β-D-galactopyranoside and allyl-N-acetyl-β-D-glucosaminide were synthesized according to the protocol described by Lee and Lee (1974).

1.7. Identification and Structural Characterization of the Oligosaccharides

The mass spectra were acquired using a mass spectrometer (Nermag R-1010C). For each experiment, the initial matrix volume is 4 µl. The products were analyzed in FAB^+ mode.

The NMR spectra were obtained using a Brucker AC300 spectrometer.

1.8. Construction of the Strain JM 107-nanA-

A JM 107 strain incapable of metabolizing sialic acid was prepared by insertional inactivation of the nana gene (Nan

operon) encoding NeuAc aldolase (Plumbridge et al., 1999). Two PCR amplification reactions were performed on either side of the center of the nanA gene so as to insert therein a BamHI restriction site.

A first 1.6-kb BamHI-XbaI fragment comprising the right-hand portion of nanA was amplified from the genomic DNA of JM 109 using the primers AAAGGATCCAAGATCAGGATGTTCACG (SEQ ID NO: 5) and GCTCTAGAATGGTAATGATGAGGCAC (SEQ ID NO: 6) and cloned between the BamHI and XbaI sites of the vector pUC19, forming the vector pUC-nan1.6. A second 2.1-kb KpnI-BamHI fragment comprising the left-hand portion of nanA was amplified using the primers AAAGGATCCGCGTAGGTGCGCTGAAAC (SEQ ID NO: 7) and AAAGGTACCTCAGGCCACCGTTAGCAG (SEQ ID NO: 8) and cloned between the KpnI and BamHI sites of the vector pUC-nan1.6, forming the vector pUC-nan3.7. The kanamycin-resistance gene (pUC-4K, Pharmacia cassette) was then cloned into the BamHI site of pUC-nan3.7. The 4.9-kb SacI-XbaI fragment containing nana::kan was inserted into the same sites of the suicide vector pCVD442 (Donnenberg and Kaper, 1991). This plasmid was used to obtain by homologous recombination JM 107 nanA::kan mutants, selected for their resistance to kanamycin and their inability to metabolize sialic acid (strain JM 107-nanA$^-$).

1.9. Construction of the Strain JM 107col$^-$DE3

Suppression of the capacity to synthesize colanic acid was achieved by insertional inactivation of the wcaJ gene encoding a glucosyl-transferase (Stevenson et al., 1996). A 1.8-kb DNA fragment containing the wcaJ gene and adjacent DNA were amplified by PCR starting with genomic DNA from JM 109, and inserted into a vector pTOPO2.1 (Invitrogen PCR cloning kit), with the aid of the primers CCACGATCCACGTCTCTCC (SEQ ID NO: 9) (right wcaJ) and AAGCTCATATCAATATGCCGCT (SEQ ID NO: 10) (left wcaJ). It was then transferred into a pUC19 vector into the EcoRI site. The vector thus obtained was subjected to a treatment with an EcoRI methylase, allowing the subsequent addition of the kanamycin-resistance gene to the ApoI site present at the center of wcaJ. The recombinant DNA wcaJ::kan was finally transferred into the suicide vector pCVD442 allowing, by homologous recombination, the production of JM 107 genomic mutants containing the inactivated gene, selected by PCR with the aid of the primers which were used for the cloning (strain JM 107-col$^-$).

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The strain JM 107-col$^-$ was made lysogenic for the phage λDE3 using the lysogenization kit from Novagen.

Example 2

Production of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranosyl]-D-glucopyranose, (A-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-D-Glc)

The principle is illustrated by FIG. **1**. We used the strain JM 109 of *Escherichia coli* K12, into which we introduced the plasmid pCWlgtA lgtA gene. The strain JM 109 is lacZ$^-$, that is to say that it is incapable of hydrolyzing lactose. On the other hand, it is lacY$^+$, which means that it can synthesize lactose permease. The lgtA gene encodes a β-1,3-N-acetyl-glucosaminyl-transferase (LgtA), which transfers an N-acetyl-glucosamine unit onto the galactose of lactose.

The strain JM 109 (pCWlgtA) and also the JM 109 control strain were cultured at high cell density (Samain et al., 1997) on glycerol as the carbon and energy sources. After the first phase of exponential growth provided by the glycerol initially present in the medium (17.5 g/l), the growth becomes limited by the glycerol, which is then added continuously at a rate of 4.5 g.h^{-1}.l^{-1}. During this second culturing phase, 90 mg.h^{-1}.l^{-1} of lactose are introduced continuously. IPTG (isopropyl-β-D-thiogalactoside) (0.5 μM) is also injected at the start of this phase to induce the expression of the lactose permease and of the β-1,3-N-acetyl-glucosaminyl-transferase. As described in FIG. **2**, the added lactose is virtually not accumulated in the medium, indicating that the lactose is indeed internalized by the bacterial cells. A large accumulation in the culture medium of a compound containing N-acetylglucosamine (hydrolyzable GlcNAc) is observed with the strain JM 109 (pCWlgtA). The amount of hydrolyzable GlcNAc (3.8 mmol/l) produced corresponds almost stoichiometrically to the amount of lactose consumed (3.5 mmol/l), suggesting that all of the lactose internalized has been glycosylated by LgtA.

At the end of the culturing, the cells are removed by centrifugation and the oligosaccharides present in the supernatant are purified by adsorption onto active charcoal and elution with ethanol. The oligosaccharides present are then separated out according to their molecular weight, on a Biogel P4 column. A single predominant compound is found. The mass spectrometry and NMR data indicate that this compound is indeed the trisaccharide (β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-β-D-Glc) formed by the addition of a GlcNAc residue to a lactose molecule. Indeed, the mass spectrum in FAB$^+$ mode shows the presence of a quasi-molecular ion [M+H]$^+$ at m/z 546 (FIG. **3**). The ^{1}H NMR spectrum confirms the trisaccharide structure, the presence of an acetyl group and the β configuration of the two O-glycoside linkages (FIG. **4**). The ^{13}C NMR spectrum also specifies that the linkage between the GlcNAc and the galactose is indeed of 1,3 type (FIG. **5**).

Example 3

Production of lacto-N-neo-tetraose and of Polylactosamine

The principle is described in FIG. **6**. The strain of *E. coli* JM 109 was cotransformed with the two plasmids pCWlgtA and pBBlgtB bearing, respectively, the genes lgtA (used previously) and lgtB (encoding a β-1,4-galactosyl-transferase known as LgtB). The strain JM 109 (pCWlgtA, pBBlgtB) was cultured at high cell density using glucose as the growth substrate. At the start of the second phase, lactose is added at high concentration (5 g.l^{-1}) or at low concentration (1 g.l^{-1})

and 0.1 mM IPTG is added. Contrary to what was observed with the strain JM 109 (pCWlgtA), only a weak release of hydrolyzable GlcNAc into the medium is detected during the culturing of this strain. On the other hand, hydrolyzable GlcNAc is found in large amount in the bacterium (FIG. 7). When the supply of lactose is 1 $g.l^{-1}$, complete internalization of the lactose (2.9 $mmol.l^{-1}$) and a total production of bound GlcNAc of 1.45 $g.l^{-1}$ (6.5 $mmol.l^{-1}$), i.e. the incorporation of more than 2 GlcNAc molecules per acceptor lactose molecule, are observed. When the lactose is added in high concentration, the internalization is incomplete (3 $g.l^{-1}$, i.e. 8.7 $mmol.l^{-1}$) with a production of GlcNAc also of about 6.5 $mmol.l^{-1}$. In this case, the GlcNAc/lactose molar ratio is close to 1, which is coherent with the synthesis of lacto-N-neotetraose.

The purification of the intracellular oligosaccharide fraction made it possible to obtain several main compounds that are well separated by chromatography on Biogel P4. The mass spectrometry and NMR data indicate that these compounds correspond to the following structures: lacto-N-neotetraose $[M+H]^+=708$; lacto-N-neo-hexaose $[M+H]^+=708$; lacto-N-neo-octaose $[M+Na]^+=1460$ and probably lacto-N-neo-decaose. The respective proportions of these various compounds depend on the amount of lactose added. Thus, with 5 $g.l^{-1}$ of lactose, the major product is lacto-N-neotetraose (FIG. 8A). On the other hand, a lower supply of lactose (1 $g.l^{-1}$) promotes the formation of compounds with a higher degree of polymerization, lacto-N-neo-octaose becoming the major product (FIG. 8B).

The formation of higher polylactosamine homologs of lacto-N-neo-tetraose is explained by the fact that LgtA is capable of using lacto-N-neo-tetraose to form an intermediate pentasaccharide that is glycosylated by LgtB to give lacto-N-neo-hexaose. The latter compound is itself the precursor for a new glycosylation cycle resulting in the formation of lacto-N-neo-octaose, and so on upto lacto-N-neo-decaose.

No significant formation of oligosaccharides with an odd number of residues and bearing a galactose in a nonreducing end position is observed. This indicates that the elongation of the molecules is limited by the incorporation of GlcNAc by LgtA rather than by the galactosylation catalyzed by LgtB.

Example 4

Production of allyl 3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside, (β-D-GlcNAc-[1→3]-β-D-Gal-1→O-allyl)

The strain JM 109(pCWlgtA) was cultured at high cell density on glycerol. At the start of the second phase of culturing, 0.75 $g.l^{-1}$ of allyl-β-D-galactopyranoside and 0.1 mM of IPTG are added. A total internalization of the allyl-β-D-galactopyranoside is observed after 9 hours, with a stoichiometric appearance of hydrolyzable GlcNAc in the extracellular medium. The oligosaccharides present in the extracellular medium are purified as in Example 2. The mass spectrum in FAB+mode of the major product obtained shows the presence of a quasi-molecular ion $[M+H]^+$ at m/z 424 corresponding to the structure β-D-GlcNAc-[1→3]-β-D-Gal-A→O-allyl.

Example 5

Production of β-D-Gal-[1→4]-β-D-GlcNAc-1→O-allyl

The strain JM 109 (pBBlgtB) was cultured at high cell density on glycerol. At the start of the second phase of culturing, 0.5 $g.l^{-1}$ of allyl-N-acetyl-β-D-glucosaminide (β-D-GlcNAc-1→O-allyl) is added. An approximately 30% reduction in the amount of extracellular hydrolyzable GlcNAc is observed in the first five hours, which demonstrates a partial internalization of allyl-N-acetyl-β-D-glucosaminide. In parallel, an almost stoichiometric intracellular production of hydrolyzable GlcNAc and of β-linked galactose residues (hydrolyzable with β-galactosidase) is observed. These results demonstrate that 30% of the allyl-N-acetyl-β-D-glucosaminide initially added has been galactosylated by the activity encoded by the lgtB gene. After purification, the structure of the expected compound (β-D-Gal-[1→4]-β-D-GlcNAc-1→O-allyl) was confirmed by mass spectrometry and NMR.

Example 6

Production of Analogs of lacto-N-neo-tetraose and of Polylactosamines in Which the Glucose Residue is Replaced with an Allyl Group

The strain JM 109 (pCWlgtA and pBBlgtB) was cultured as in Example 3, except that the supply of lactose was replaced with the addition of 0.65 $g.l^{-1}$ of allyl-β-D-galactopyranoside. After purification according to the protocol of Example 3, three main compounds are obtained. The mass spectrometry data indicate that these three compounds correspond to the tri-, penta- and heptasaccharides below:

β-D-Gal-[1→4]-β-D-GlcNAc-[1→3]-β-D-Gal-1→O-allyl, $[M+H]=586$;

β-D-Gal-[1→4]-β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-D-GlcNAc-[1→3]-β-D-Gal-1→O-allyl, $[M+H]=951$;

β-D-Gal-[1→4]-β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-β-D-GlcNAc-[1→3]-β-D-Gal-1→O-allyl, $[M+H]=1316$.

Example 7

Production of 3'-sialyllactose (α-NeuAc-[2→3]-β-D-Gal-[1→4]-β-D-Glc)

The principle is illustrated by FIG. 9. The genes for the biosynthesis of sialic acid and CMP-NeuAc are not present in *E. coli* K12. However, *E. coli* K12 is capable of degrading sialic acid (Plumbridge and Vimr, 1999) and contains a permease (NanT) that enables exogenous sialic acid to enter into the cell. This sialic acid is then normally catabolized by an aldolase (NanA).

We used the strain of *Escherichia coli* K12 JM 107-nanA$^-$ (Example 1) and a JM 107 control strain into which we introduced the two compatible plasmids NST-01 and pBBnsy containing, respectively, the genes for α-2,3-sialyl-transferase and for CMP-NeuAc synthase. This strain lacks nanA activity and is thus incapable of degrading intracellular sialic acid. However, it contains the lactose permease (lacy) and sialyl permease (nanT) genes and is thus capable of internalizing exogenous sialic acid and lactose. The internalized sialic acid may thus be activated to CMP-NeuAc by the action

of CMP-NeuAc synthase and transferred onto the intracellular lactose by the action of α-2,3-sialyl-transferase.

The strain JM 107-nanA⁻ (Nst-01, pBBnsy) and the JM 107 control strain (Nst-01, pBBnsy) having the NanA activity were cultured at high cell density on glycerol. Lactose (1.5 $g.l^{-1}$), IPTG (0.1 mM) and sialic acid (0.6 $g.l^{-1}$) are added at the start of the second phase of culturing for a period of five hours. Throughout the duration (17 hours) of the third phase of culturing, 100 $mg.h^{-1}.L^{-1}$ of sialic acid and 200 $mg.h^{-1}L^{-1}$ of lactose are introduced continuously.

At the end of culturing of the strain JM 107-nanA⁻(Nst-01, pBBnsy), enzymatic assay of the lactose with and without treatment with a neuraminidase makes it possible to estimate the total production of sialyllactose as 2.6 $g.l^{-1}$. This production is partly found in the bacterial cells (1.5 $g.l^{-1}$) and in the extracellular culture medium (1.1 $g.l^{-1}$). In the case of the JM 107 control strain (Nst-01, pBBnsy), the production of sialyllactose is much lower (150 $mg.l^{-1}$), indicating that virtually all of the sialic acid has been degraded by the bacterium.

The intracellular and extracellular oligosaccharides are purified by adsorption onto active charcoal and elution with ethanol. After purification on an anion-exchange resin, only one product is detected by HPLC.

The mass spectrum in FAB^+ mode shows the presence of two quasi-molecular ions [M+H]+at m/z 656 and [M+Na] at m/z 678 corresponding to the sodium salt of sialyllactose.

Example 8

Production of Fucosyl Derivatives of lacto-N-neo-tetraose

In *E. coli* K12, the genes for the biosynthesis of GDP-fucose form part of the operon responsible for the biosynthesis of an extracellular polysaccharide, colanic acid (Stevenson et al., 1996). The expression of this operon is controlled by a complex regulation network in which the protein RcsA is involved (Stout et al., 1991). The overexpression of the rcsA gene is thus reflected by an overproduction of colanic acid (Russo and Singh, 1993) and consequently of the genes for the biosynthesis of GDP fucose.

To increase the availability of GDP fucose, our strategy consisted in using a strain of *E. coli* in which the rcsA gene was overexpressed (so as to overproduce the genes for the biosynthesis of GDP-fucose) and in which one of the genes that is essential to the biosynthesis of colanic acid has been inactivated (so as to totally suppress the production of colanic acid and to avoid a competition for the use of GDP-fucose).

We used the strain JM 107-col⁻DE3 in which the wcaJ gene, which is responsible for the transfer of the first glucose residue of the repeating unit, has been inactivated according to Example 1 and into which we introduced either the two plasmids pHP0651 and pBBLnt, or the two plasmids pHP0651 and pBBLntRcsA. The plasmid pHP0651 contains the fucT gene for α-1,3-fucosyl-transferase of *Helicobacter pylori*. This fucosyl-transferase uses as acceptor N-acetyllactosamine and lacto-N-neo-tetraose, but not lactose (Martin et al., 1997). The plasmid pBBLnt contains the lgtA and lgtB genes. The plasmid pBBLntRcsA contains the lgtA, lgtB and rcsA genes.

The two strains JM 107-col⁻DE3 (pHP0651, pBBLnt) and JM 107-col⁻DE3 (pHP0651, pBBLntRcsA) were cultured as in Example 3 in the presence of 5 $g.l^{-1}$ of lactose. At the end of the third phase of culturing, the amount of hydrolyzable GlcNAc produced by the two strains (1.7 $g.l^{-1}$) was comparable to that obtained with the strain JM 109 (pCWlgtA, pBBlgtB) in Example 3. The colorimetry assay of the fucose at the end of culturing shows a large difference between the two strains, with a fucose production of 1 $g.l^{-1}$ for the strain JM 107-col⁻DE3 (pHP0651, pBBLntRcsA) and only 0.25 $g.l^-$ for the strain JM 107-col⁻DE3 (pHP0651, pBBLnt). The fucosyl oligosaccharides are found at more than 70% in the intracellular fraction.

Purification of the intracellular fraction by adsorption onto active charcoal and steric exclusion chromatography on Biogel P2 makes it possible to separate four main compounds.

Compound 1 corresponds, by its elution volume on Biogel P2 and its thin-layer migration, to lacto-N-neo-tetraose.

The mass spectrum of compound 2 shows the presence of a quasi-molecular ion $[M+H]^+$at m/z 854 corresponding to the molar mass of lacto-N-fucopentaose. The presence of a secondary ion at 327 indicates that the molecule is fucosylated on the glucose residue and has the following structure β-D-Gal-[1→4]-β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-(α-L-Fuc-[1→3])-β-D-Glc.

The mass spectrum of the major compound 3 shows the presence of 3 quasi-molecular ions at m/z 1000, 1022 and 1038 corresponding to the three forms $[M+H]^+$, [M+Na]+ and [M+K]+ of the lacto-N-difucohexaose molecule having the following structure β-D-Gal-[1→4]-4-(β-L-Fuc-[1→3])-β-D-GlcNAc-[1→3])-β-D-Gal-[1→4]-(α-L-Fuc-[1→3])-β-D-Glc.

The mass spectrum of compound 4 makes it possible to identify two quasi-molecular ions at m/z 1365 and 1388 corresponding to the $[M+H]^+$ and $[M+Na]^+$ forms of a lacto-N-difucooctaose molecule. The presence of a secondary ion at m/z 512 indicates that the GlcNAc residue of the nonreducing end bears a fucose. The NMR data show that the ^1H proton of a fucose residue is sensitive to anomerism and that this fucose residue is thus bound to the glucose. These results make it possible to propose the following structure for compound 4: β-D-Gal-[1→4]-(α-L-Fuc-[1→3])-β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-β-D-GlcNAc-[1→3]-β-D-Gal-[1→4]-(α-L-Fuc-[1→3])-β-D-Glc.

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The invention claimed is:

1. A method for producing an oligosaccharide comprising lactose using a genetically modified cell starting with at least one internalized exogenous precursor consisting of lactose said method comprising:

(i) obtaining a Lac Z^-Y^+ *E. coli* cell that

comprises at least one recombinant gene encoding an enzyme capable of modifying said exogenous precursor or one of the intermediates in the biosynthetic pathway of said oligosaccharide from said exogenous precursor necessary for the synthesis of said oligosaccharide from said exogenous precursor, and also the components for expressing said gene in said cell; and

(ii) culturing said cell on a carbon-based substrate in the presence of at least one said exogenous precursor, under conditions inducing the internalization according to a mechanism of active transport of said exogenous precursor by said cell and the production of said oligosaccharide by said cell,

wherein said culturing comprises:

(a) a first phase of exponential cell growth ensured by said carbon-based substrate,

(b) a second phase of cell growth limited by said carbon-based substrate which is added continuously,

wherein said precursor is added during the second phase.

2. The method as claimed in claim 1, wherein said modification is selected from the group consisting of glycosylation, sulfation, acetylation, phosphorylation, succinylation, methylation, and addition of an enolpyruvate group.

3. The method as claimed in claim 1, wherein said enzyme is an enzyme capable of performing a glycosylation, chosen from glycosyl-transferases.

4. The method as claimed in claim 3, wherein said enzyme is a glycosyl-transferase selected from the group consisting of β-1,3-N-acetyl-glucosaminyl-transferase, β-1,3-galactosyl-transferase, β-1,3-N-acetyl-galactosaminyl-transferase, β-1,3-glucuronosyl-transferase, β-1,3-N-acetyl-galactosaminyl-transferase, β-1,4-N-acetyl-galactosaminyl-transferase, β-1,4-galactosyl-transferase, α-1,3-galactosyl-transferase, α-1,4-galactosyl-transferase, α-2,3-sialyl-transferase, α-2,6-sialyl-transferase, α-2,8-sialyl-transferase, α-1,2-fucosyl-transferase, α-1,3-fucosyl-transferase and α-1,4-fucosyl-transferase.

5. The method as claimed in claim 1, wherein said carbon-based substrate is selected from the group consisting of glycerol and glucose.

6. The method as claimed in claim 1, wherein said culturing is performed under conditions allowing the production of a culture with a high cell density.

7. The method as claimed in claim 6, wherein said culturing further comprises

(c) a third phase of slowed cell growth obtained by continuously adding to the culture an amount of said substrate that is less than the amount of substrate added in said second phase so as to increase the content of oligosaccharides produced in the high cell density culture.

8. The method as claimed in claim 7, wherein the amount of substrate added continuously to the cell culture during said third phase is at least 30% less than the amount of substrate added continuously during said second phase.

9. The method as claimed in claim 1, further comprising the addition of an inducer to said culture medium to induce the expression in said cell of said enzyme and/or of a protein involved in said transport.

10. The method as claimed in claim 9, wherein said inducer is isopropyl β-D-thiogalactoside (IPTG) and said protein is lactose permease.

11. The method as claimed in claim 1, for the production of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranosyl]-D-glucopyranose, (β-D-GlcNac-[1→3]-β-D-Gal-[1→4]-D-Glc), wherein:

said cell is a bacterium of $LacZ^-$, $LacY^+$ genotype;

said enzyme is β-1,3-N-acetyl-glucosaminyl-transferase;

said substrate is glycerol;

said inducer is isopropyl β-D-thiogalactoside (IPTG); and

said precursor is lactose.

12. The method as claimed in claim 1, for the production of lacto-N-neo-tetraose and polylactosamine (lacto-N-neo-hexaose, lacto-N-neo-octaose, lacto-N-neo-decaose); further comprising the addition of an inducer to said culture medium to induce the expression in said cell of said enzyme and/or of a protein involved in said transport-wherein:

said cell is a bacterium of $LacZ^-$, $LacY^+$ genotype;

said enzymes are β-1,3-N-acetyl-glucosaminyl-transferase and β-1,4-galactosyl-transferase;

said inducer is isopropyl-β-D-thiogalactoside (IPTG); and

said precursor is lactose.

13. The method as claimed in claim 1, wherein said cell is cultured on said carbon-based substrate labeled with at least one isotope or in the presence of said exogenous precursor labeled with said isotope.

14. A method for producing an oligosaccharide comprising lactose by a genetically modified cell starting with at least one internalized exogenous precursor consisting of lactose, said method comprising:

(i) obtaining a Lac Z^-Y^+ *E. coli* cell comprising (a) at least one recombinant gene encoding an enzyme capable of modifying said exogenous precursor, and (b) the components for expressing said gene in said cell;

(ii) culturing said cell on a carbon-based substrate in the presence of at least one said exogenous precursor and lactose permease, under conditions inducing the internalization according to a mechanism of active transport of said exogenous precursor by said cell and the production of said oligosaccharide by said cell,

wherein the enzyme is a glycosyl-transferase selected from the group consisting of β-1,3-N-acetyl-glucosaminyl-transferase, β-1,3-galactosyl-transferase, β-1,3-N-acetyl-galactosaminyl-transferase, β-1,3-glucuronosyl-transferase, β-1,3-N-acetyl-galactosaminyl-transferase, β-1,4-N-acetyl-galactosaminyl-transferase, β-1,4-galactosyl-transferase, α-1,3-galactosyl-transferase, α-1,4-galactosyl-transferase, α-2,3-sialyl-transferase, α-2,6-sialyl-transferase, α-2,8-sialyl-transferase, α-1,2-fucosyl-transferase, α-1,3-fucosyl-transferase and α-1,4-fucosyl-transferase, and

wherein said culturing comprises:

(a) a first phase of exponential cell growth ensured by said carbon-based substrate;

(b) a second phase of cell growth limited by said carbon-based substrate which is added continuously; and

wherein said precursor is added during the second phase.

* * * * *

EXHIBIT B

UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF COLUMBIA

WYETH, *et al.*,	:	
	:	
Plaintiffs,	:	
	:	
v.	:	Civil Action No. 07-1492 (JR)
	:	
JON W. DUDAS, Under Secretary of Commerce for Intellectual Property and Director of U.S. Patent and Trademark Office,	:	
	:	
Defendant.	:	

MEMORANDUM OPINION

Plaintiffs here take issue with the interpretation that the United States Patent and Trademark Office (PTO) has imposed upon 35 U.S.C. § 154, the statute that prescribes patent terms. Section 154(a)(2) establishes a term of 20 years from the day on which a successful patent application is first filed. Because the clock begins to run on this filing date, and not on the day the patent is actually granted, some of the effective term of a patent is consumed by the time it takes to prosecute the application. To mitigate the damage that bureaucracy can do to inventors, the statute grants extensions of patent terms for certain specified kinds of PTO delay, 35 U.S.C. § 154(b)(1)(A), and, regardless of the reason, whenever the patent prosecution takes more than three years. 35 U.S.C. § 154(b)(1)(B). Recognizing that the protection provided by these separate guarantees might overlap, Congress has forbidden double-counting: "To the extent that periods of delay attributable to grounds

specified in paragraph (1) overlap, the period of any adjustment granted under this subsection shall not exceed the actual number of days the issuance of the patent was delayed." 35 U.S.C. § 154(b)(2)(A). Plaintiffs claim that the PTO has misconstrued or misapplied this provision, and that the PTO is denying them a portion of the term Congress has provided for the protection of their intellectual property rights.

Statutory Scheme

Until 1994, patent terms were 17 years from the date of issuance. See 35 U.S.C. § 154 (1992) ("Every patent shall contain . . . a grant . . . for the term of seventeen years . . . of the right to exclude others from making, using, or selling the invention throughout the United States. . . ."). In 1994, in order to comply with treaty obligations under the General Agreement on Tarriffs and Trade (GATT), the statute was amended to provide a 20-year term from the date on which the application is first filed. See Pub. L. No. 103-465, § 532, 108 Stat. 4809, 4984 (1994). In 1999, concerned that extended prosecution delays could deny inventors substantial portions of their effective patent terms under the new regime, Congress enacted the American Inventors Protection Act, a portion of which -- referred to as the Patent Term Guarantee Act of 1999 -- provided for the adjustments that are at issue in this case. Pub. L. No. 106-113, §§ 4401-4402, 113 Stat. 1501, 1501A-557 (1999).

As currently codified, 35 U.S.C. § 154(b) provides three guarantees of patent term, two of which are at issue here. The first is found in subsection (b)(1)(A), the "[g]uarantee of prompt Patent and Trademark Office response." It provides a one-day extension of patent term for every day that issuance of a patent is delayed by a failure of the PTO to comply with various enumerated statutory deadlines: fourteen months for a first office action; four months to respond to a reply; four months to issue a patent after the fee is paid; and the like. See 35 U.S.C. § 154(b)(1)(A)(i)-(iv). Periods of delay that fit under this provision are called "A delays" or "A periods." The second provision is the "[g]uarantee of no more than 3-year application pendency." Under this provision, a one-day term extension is granted for every day greater than three years after the filing date that it takes for the patent to issue, regardless of whether the delay is the fault of the PTO.[1] See 35 U.S.C. § 154(b)(1)(B). The period that begins after the three-year window has closed is referred to as the "B delay" or the "B period". ("C delays," delays resulting from interferences, secrecy orders, and appeals, are similarly treated but were not involved in the patent applications underlying this suit.)

---

[1] Certain reasons for exceeding the three-year pendency period are excluded, see 35 U.S.C. § 154(b)(1)(b)(i)-(iii), as are periods attributable to the applicant's own delay. See 35 U.S.C. § 154(b)(2)(C).

The extensions granted for A, B, and C delays are subject to the following limitation:

> **(A) In general.**--To the extent that periods of delay attributable to grounds specified in paragraph (1) overlap, the period of any adjustment granted under this subsection shall not exceed the actual number of days the issuance of the patent was delayed.

35 U.S.C. § 154(b)(2)(A). This provision is manifestly intended to prevent double-counting of periods of delay, but understanding that intent does not answer the question of what is double-counting and what is not. Proper interpretation of this proscription against windfall extensions requires an assessment of what it means for "periods of delay" to "overlap."

The PTO, pursuant to its power under 35 U.S.C. § 154(b)(3)(A) to "prescribe regulations establishing procedures for the application for and determination of patent term adjustments," has issued final rules and an "explanation" of the rules, setting forth its authoritative construction of the double-counting provision. The rules that the PTO has promulgated essentially parrot the statutory text, see 37 C.F.R. § 1.703(f), and so the real interpretive act is found in something the PTO calls its Explanation of 37 CFR 1.703(f) and of the United States Patent and Trademark Office Interpretation of 35 U.S.C. § 154(b)(2)(A), which was published on June 21, 2004, at 69 Fed. Reg. 34238. Here, the PTO "explained" that:

> the Office has consistently taken the position that if an application is entitled to an adjustment under the three-year pendency provision of 35 U.S.C. § 154(b)(1)(B), the entire period during which the application was pending before the Office (except for periods excluded under 35 U.S.C. § 154(b)(1)(B)(i)-(iii)), and not just the period beginning three years after the actual filing date of the application, is the relevant period under 35 U.S.C. § 154(b)(1)(B) in determining whether periods of delay "overlap" under 35 U.S.C. 154(b)(2)(A).

69 Fed. Reg. 34238 (2004) (emphasis added). In short, the PTO's view is that any administrative delay under § 154(b)(1)(A) overlaps any 3-year maximum pendency delay under § 154(b)(1)(B): the applicant gets credit for "A delay" or for "B delay," whichever is larger, but never A + B.

In the plaintiffs' submission, this interpretation does not square with the language of the statute. They argue that the "A period" and "B period" overlap only if they occur on the same calendar day or days. Consider this example, proffered by plaintiff: A patent application is filed on 1/1/02. The patent issues on 1/1/08, six years later. In that six-year period are two "A periods," each one year long: (1) the 14-month deadline for first office action is 3/1/03, but the first office action does not occur until 3/1/04, one year late; (2) the 4-month deadline for patent issuance after payment of the issuance fee is

1/1/07, but the patent does not issue until 1/1/08, another year of delay attributable to the PTO. According to plaintiff, the "B period" begins running on 1/1/05, three years after the patent application was filed, and ends three years later, with the issuance of the patent on 1/1/08. In this example, then, the first "A period" does not overlap the "B period," because it occurs in 2003-04, not in 2005-07. The second "A period," which covers 365 of the same days covered by the "B period," does overlap. Thus, in plaintiff's submission, this patent holder is entitled to four years of adjustment (one year of "A period" delay + three years of "B period" delay). But in the PTO's view, since "the entire period during which the application was pending before the office" is considered to be "B period" for purposes of identifying "overlap," the patent holder gets only three years of adjustment.

Chevron Deference

We must first decide whether the PTO's interpretation is entitled to deference under Chevron v. NRDC, 467 U.S. 837 (1984). No, the plaintiffs argue, because, under the Supreme Court's holdings in Gonzales v. Oregon, 546 U.S. 243 (2006), and United States v. Mead Corp., 533 U.S. 218 (2001), Congress has not "delegated authority to the agency generally to make rules carrying the force of law," and in any case the interpretation at issue here was not promulgated pursuant to any such authority.

See Gonzales, 546 U.S. at 255-56, citing Mead, 533 U.S. at 226-27. Since at least 1996, the Federal Circuit has held that the PTO is not afforded Chevron deference because it does not have the authority to issue substantive rules, only procedural regulations regarding the conduct of proceedings before the agency. See Merck & Co. v. Kessler, 80 F.3d 1543, 1549-50 (Fed. Cir. 1996).

Here, as in Merck, the authority of the PTO is limited to prescribing "regulations establishing procedures for the application for and determination of patent term adjustments under this subsection." 35 U.S.C. § 154(b)(3)(A) (emphasis added). Indeed, a comparison of this rulemaking authority with the authority conferred for a different purpose in the immediately preceding section of the statute makes it clear that the PTO's authority to interpret the overlap provision is quite limited. In 35 U.S.C. § 154(b)(2)(C)(iii) the PTO is given the power to "prescribe regulations establishing the circumstances that constitute a failure of an applicant to engage in reasonable efforts to conclude processing or examination of an application" (emphasis added) -- that is, the power to elaborate on the meaning of a particular statutory term. No such power is granted under § 154(b)(3)(A). Chevron deference does not apply to the interpretation at issue here.

Statutory Construction

Chevron would not save the PTO's interpretation, however, because it cannot be reconciled with the plain text of the statute. If the statutory text is not ambiguous enough to permit the construction that the agency urges, that construction fails at Chevron's "step one," without regard to whether it is a reasonable attempt to reach a result that Congress might have intended. See, e.g., MCI v. AT&T, 512 U.S. 218, 229 (1994) ("[A]n agency's interpretation of a statute is not entitled to deference when it goes beyond the meaning that the statute can bear.").

The operative question under 35 U.S.C. § 154(b)(2)(A) is whether "periods of delay attributable to grounds specified in paragraph (1) overlap." The only way that periods of time can "overlap" is if they occur on the same day. If an "A delay" occurs on one calendar day and a "B delay" occurs on another, they do not overlap, and § 154(b)(2)(A) does not limit the extension to one day. Recognizing this, the PTO defends its interpretation as essentially running the "period of delay" under subsection (B) from the filing date of the patent application, such that a period of "B delay" always overlaps with any periods of "A delay" for the purposes of applying § 154(b)(2)(A).

The problem with the PTO's construction is that it considers the application delayed under § 154(b)(1)(B) during the

period before it has been delayed. That construction cannot be squared with the language of § 154(b)(1)(B), which applies "if the issue of an original patent is delayed due to the failure of the United States Patent and Trademark Office to issue a patent within 3 years." (Emphasis added.) "B delay" begins when the PTO has failed to issue a patent within three years, not before.

The PTO's interpretation appears to be driven by Congress's admonition that any term extension "not exceed the actual number of days the issuance of the patent was delayed," and by the PTO's view that "A delays" during the first three years of an applications' pendency inevitably lead to "B delays" in later years. Thus, as the PTO sees it, if plaintiffs' construction is adopted, one cause of delay will be counted twice: once because the PTO has failed to meet and administrative deadline, and again because that failure has pushed back the entire processing of the application into the "B period." Indeed, in the example set forth above, plaintiffs' calendar-day construction does result in a total effective patent term of 18 years under the (B) guarantee, so that - again from the PTO's viewpoint -- the applicant is not "compensated" for the PTO's administrative delay, he is benefitted by it.

But if subsection (B) had been intended to guarantee a 17-year patent term and no more, it could easily have been written that way. It is true that the legislative context -- as

distinct from the legislative history -- suggests that Congress may have intended to use subsection (B) to guarantee the 17-year term provided before GATT. But it chose to write a "[g]uarantee of no more than 3-year application pendency," 35 U.S.C. § 154(b)(1)(B), not merely a guarantee of 17 effective years of patent term, and do so using language separating that guarantee from a different promise of prompt administration in subsection (A). The PTO's efforts to prevent windfall extensions may be reasonable -- they may even be consistent with Congress's intent -- but its interpretation must square with Congress's words. If the outcome commanded by that text is an unintended result, the problem is for Congress to remedy, not the agency.

JAMES ROBERTSON
United States District Judge

UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF COLUMBIA

WYETH, *et al.*,	:	
	:	
Plaintiffs,	:	
	:	
v.	:	Civil Action No. 07-1492 (JR)
	:	
JON W. DUDAS, Under Secretary of Commerce for Intellectual Property and Director of U.S. Patent and Trademark Office,	:	
	:	
Defendant.	:	

ORDER

For the reasons stated in the accompanying memorandum opinion, plaintiffs' motion for summary judgment [12] is **GRANTED** and defendant's motion for summary judgment [16] is **DENIED**. The case is remanded to the agency for further proceedings that are consistent with this opinion.

JAMES ROBERTSON
United States District Judge

EXHIBIT C

CLOSE WINDOW ALL CASES SELECT CASE

Patent Term Adjustment Calculation System

Add a new event to this case

Docket Number: 065691-0267
Application Number: 10/019954
Patent Number: N/A

	Event Description	Event Date	Days from Filing	PTO Days	Applicant Days
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Edit Delete	International Filing Date	07/07/2000	-549		
	PCT National Stage Commencement Date	01/07/2002	0		
Edit Delete	National Stage Entry (All 371(c) Requirements Met)	05/24/2002	137		
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	3 Year Period Starts	01/07/2005	1,096		
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